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CERAMIDE-ENRICHED EXTRACELLULAR VESICLES: A ROLE IN ENHANCING AMYLOID-BETA NEUROTOXICITY AND MITOCHONDRIAL DAMAGE IN ALZHEIMER'S DISEASE

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine
at the University of Kentucky

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ABSTRACT OF DISSERTATION

CERAMIDE-ENRICHED EXTRACELLULAR VESICLES: A ROLE IN ENHANCING AMYLOID-BETA NEUROTOXICITY AND MITOCHONDRIAL DAMAGE IN ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is an age-dependent, progressive, neurodegenerative disorder that is characterized clinically by the impairment of cognitive functions concomitant with behavioral and personality changes. AD is associated with distinct pathological hallmarks, namely, intracellular neurofibrillary tangles comprised of hyperphosphorylated tau protein, extracellular amyloid beta (Aβ) plaques, and marked brain atrophy. Besides their main role as the core component of amyloid plaques, oligomeric Aβ have been shown to be neurotoxic. The exact mechanism of Aβ neurotoxicity is yet to be elucidated.

Recently, a pathogenic function of small extracellular vesicles- also known as exosomes- has been proposed, suggesting that exosomes can transfer pathogens between cells. One such pathogen that exploits this pathway is Aβ in Alzheimer's disease, however, it is not known yet whether this Aβ/exosomes association would affect the neuronal toxicity of Aβ.

Exosomes are nano-sized lipid vesicles that are formed by inward budding of late endosomes to form multi vesicular bodies (MVB) which fuse to the plasma membrane and release exosomes to the extracellular space. Exosomes serve as a means of intercellular communication due to their ability in carrying cargoes including microRNA (miRNA), messenger RNA (mRNA), proteins, and other biomolecules. There are several established pathways for exosomes biogenesis, one of which is triggered by the sphingolipid ceramide. Ceramide is a key molecule in sphingolipids metabolism and it is involved in several cellular processes such as proliferation, senescence and apoptosis. It has also been reported that ceramide levels are elevated in AD patients brain specimens.

Exploiting the fact that exosomes can cross the blood brain barrier we therefore used serum derived exosomes to study the biophysical and biochemical characteristics of Alzheimer's disease mouse model (5xFAD) and AD patients' exosomes compared to wild type and healthy individuals. We found that serum from 5xFAD mice and AD patients contain a subpopulation of astrocyte-derived exosomes that are enriched with ceramide,

particularly C16:0, C18:0, C20:0, 22:0, C24:0, and C24:1 ceramide species. This subpopulation (termed astrosomes) was shown to associated with Aβ and are prone to aggregation as confirmed by nanoparticle tracking and cluster analyses. To study the functional characteristics of these Aβ-associated astrosomes, we used Neuro-2a (N2a) cells, human iPS cell-derived neurons, and mouse primary cultured neurons as *in vitro* tissue culture models. When taken up by neurons, Aβ-associated astrosomes were specifically transported to mitochondria where they induced mitochondria clustering, evident by elevation of expression of the fission protein dynamin related protein1 (Drp1). Aβ-associated astrosomes, but not wild type or healthy control human exosomes, mediated binding of Aβ to voltage-dependent anion channel 1 (VDAC1), a gate keeper protein in the outer mitochondrial membrane that is involved in regulating passage of metabolites, nucleotides, and ions; it plays a crucial role in regulating apoptosis. This Aβ/VDAC1 interaction leads to caspase activation and subsequently apoptosis. Interestingly, removing the ceramide-enriched astrosomes from the exosome pool using lipid-mediated affinity chromatography (LIMAC) mitigated that toxic effect on neurons. These results were replicated using brain derived exosomes.

To investigate the *in vivo* significance of our *in vitro* results, we stereotaxically injected wild type mice (two weeks old) with 5xFAD or wild type brain derived exosomes (nine months old). We found that within two days, the injected exosomes were specifically taken up by neurons and transported to mitochondria. Consistent with our *in vitro* data using Aβ-associated astrosomes, the exosomes isolated from 5xFAD brain, but not those from wild type brain, induced complex formation of Aβ with VDAC1 and activation of caspase 3.

To test that our observations hold true in physiological conditions, we generated a novel astrosome reporter mouse model. This was accomplished by crossing of Aldh1l1-Cre/ERT with floxed CD63-GFP and 5xFAD mice (5XFAD xAldh1l1-Cre/ERTxCD63-$GFP^{fl/fl}$) which allows us to track astrosome uptake and their subsequent effects. As seen with the injected exosomes, we found that endogenous GFP-labeled astrosomes are taken up by neurons where they shuttle Aβ and induce mitotoxicity.

In conclusion, our data show that association of Aβ to astrosomes in critical for Aβ neurotoxicity. Therefore, we discovered a novel mechanism by which Aβ induces AD neuropathology as well as potential pharmacological target.

KEYWORDS: Alzheimer's disease, exosomes, ceramide, astrocytes, sphingolipids.

Ahmed Elsherbini

10/14/2020

Date

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CHAPTER 1. INTRODUCTION

1.1 Alzheimer's Disease

1.1.1 Background

Dementia is a general term that encompasses a severe set of symptoms which can interfere with a person's daily life and activities [1]. These symptoms include thinking and social abilities as well as changes in memory. Furthermore, dementia could result in a functional decline in memory, visual perception, language skills, self-management, problem solving, and one's ability to focus or pay attention [2].

Dementia comprises a wide array of specific medical conditions, rather than one sole entity. There are several causes of dementia including vascular cognitive impairment, dementia with Lewy bodies, Frontotemporal dementia, and Parkinson's disease [2, 3]. Alzheimer's disease (AD) accounts for 60-80% of all dementia cases [4]. Alois Alzheimer, a German psychiatrist, presented the first signature case of AD, in 1906, at the 37th convention of Southwestern German Psychiatrist [5]. The disease did not coin the name "Alzheimer's" until 1910, when Emil Kraepelin, a coworker of Alois Alzheimer, named the disease after him in honor of his achievements.

Despite the heterogeneity of AD symptoms among patients, the most common initial symptom is a gradual worsening ability to remember new information. Other common cognitive related symptoms include memory loss that results in daily life disturbance, struggle with words in speaking or writing, challenges in planning or solving problems, misplacing things and losing the ability to retrace steps, and not being able to complete familiar tasks at home, at work or at leisure[4, 6]. The list of symptoms can occur in unison with confusion, involving time and place, and difficulty understanding visual

images and spatial relationships. AD patients suffer from non-cognitive symptoms as well. These symptoms include, but are not limited to, decreased or poor judgment, sudden changes in mood and personality, trouble keeping up with social activities, increased anxiety, agitation, and sleep disturbances.

With respect to diagnosis, there is currently no distinct test for Alzheimer's disease. Rather, it takes a consultation between physicians and neurologists utilizing different approaches and methods to reach a diagnosis. The patients' medical and family history should be examined, as well as their psychiatric history and a history of cognitive and behavioral changes. Medical personnel should confer with family members or individuals close to the patient, to provide input on changes in thinking skills or behavior. Cognitive tests along with physical and neurological examinations may be conducted as well. In order to affirm the diagnosis, the patient may undergo blood tests and brain imaging. Testing can rule out other potential causes of dementia symptoms, such as a tumor or particular vitamin deficiencies. Diagnosing Alzheimer's disease requires a careful and comprehensive medical evaluation. Although physicians can almost always determine if a person has dementia, identifying the exact cause may pose some difficulty. Several days or weeks may be needed for the patient to complete the required tests and examinations and for the physician to interpret the results and make a diagnosis [6].

AD pathology is characterized by three distinct hallmarks: Extracellular amyloid (senile) plaques with amyloid beta (Aβ) protein being the main component, intracellular neurofibrillary tangles comprised of hyperphosphorylated tau protein, and noticeable neuronal loss [7].

The pace of the disease, how fast it progresses, differs from one patient to another, much like the symptoms. While all patients experience similar symptoms, there are two well-established types of AD. The first type is early-onset AD (EOAD) which is associated with progressive deposition of Aβ peptides in patients < 60 years of age, and it accounts for 1-5% of all AD cases [4, 8-11]. The second type is late-onset AD, which accounts for ≥ 95% of cases. Despite sharing the same pathophysiology, the etiology of EAOD and LOAD are different. While EOAD is mainly genetic, LOAD is more complex and multifactorial with several risk factors participating in the development of the disease, including genetic and environmental risk factors and most importantly aging [8].

The pathogenic Aβ is the product of proteolytic cleavage of Amyloid precursor protein (APP) [12]. APP is an integral membrane glycoprotein that is expressed in several tissues. The exact physiological role of APP is not clear, however, in the brain it is reported to contribute to neurite outgrowth, synaptic plasticity, and regulation of neuronal excitation. APP can be produced as several isoforms with a size ranging from 695 to 770 amino acids [13]. APP695 represents the most abundant form of APP in the brain and it is produced mainly in neurons. The three enzymes responsible for cleavage of APP are alpha, beta and y-secretase. This process can happen in two ways: amyloidogenic or non-amyloidogenic. Under normal non-amyloidogenic conditions (approximately 90% of APP cleavage) α-secretase first cleaves APP within the Aβ-region releasing APPα from the cell surface and an 83-amino acid C-terminal fragment (CTF)–α. CTF- α is further cleaved by γ-secretase into P3(3 KDa) and APP intracellular domain(AICD)[13, 14]. By contrast, the remaining 10% of APP is cleaved by amyloidogenic processing, which involves sequential cleavage of APP by β-secretase followed by γ-secretase. β-secretase cleavage results in

the formation of soluble APPβ and CTFβ, which is further processed by γ-secretase. Unlike the β-secretase processing site, which is precisely at amino acid 671 of APP, γ-secretase cleavage is rather imprecise and could happen in multiple sites producing fragments of 43, 45, 46, 48, 49 and 51 amino acids. These fragments are further cleaved in endocytic compartments into 40 and 42- amino acid Aβ (Aβ40 and Aβ42), the two main forms of Aβ[15]. (Fig.1.1).

The pathogenesis of AD is often attributed to the dynamics of production and clearance of Aβ, especially Aβ42. Hence, amyloid positron-emission tomography (PET) imagining as well as decreased levels of Aβ42 and/or Aβ42/Aβ40 ratio in cerebrospinal fluid (CSF) are recommended for diagnosis of AD [16].

On the other hand, neurofibrillary tangles (NFTs) are intraneuronal cytoplasmic bundles of paired, helically wound filaments comprised of hyperphosphorylated tau protein [17]. Under physiological conditions, tau modulates the stability of axonal microtubules [18]. Tau contains 85 phosphorylation sites and therefore it is not surprising that phosphorylation, at any of these sites, profoundly impacts its function. Under pathological conditions such as AD, tau becomes hyperphosphorylated, reducing its affinity for microtubules and destabilizing the cytoskeleton of the affected neuron [18]. Additionally, hyperphosphorylation of tau promotes aberrant assembly of tau into insoluble aggregates that induce synaptic dysfunction and neuronal cell death.

1.1.2 The Amyloid Cascade (amyloid beta) Hypothesis

In 1984, George Glenner was the first to propose that a particular amyloidogenic protein accumulates in AD and could be the causative of the disease pathology[19]. However, it was in 1991 (Beyreuther & Masters; Hardy & Allsop;Selkoe) when the amyloid hypothesis idea was put forward and to this day remains the dominant model of AD pathogenesis[20-22]. The hypothesis proposes that the production and excessive accumulation of Aβ, both intracellularly and extracellularly, under different aggregation and physical states, instigate a pathological progression cascade leading to neurofibrillary tangles formation, synaptotoxicity, mitochondrial dysfunction, chronic neuroinflammation arising from aberrant activation of glial cells, and ultimately neurodegeneration and dementia. Despite the lack of consensus about this hypothesis, it remains one of the most extensively validated and compelling hypothesis of AD pathogenesis[23, 24].

Several lines of evidence support the Aβ hypothesis, most notably was the discovery that humans with trisomy 21 (Down's syndrome) harbor 3 copies of APP and experience an increased risk for developing neuropathologically typical AD as they age[25]. In fact, almost all Down syndrome adults over the age of 40 exhibit neuropathology sufficient for an AD diagnosis. The APP gene is located on chromosome 21, indicating that Down's syndrome patients develop typical Alzheimer neuropathology due to the over production of Aβ throughout their lives. Interestingly, patients with partial trisomy 21, with translocation involving only the distal part of chromosome 21 telomeric to the APP gene, develop Down Syndrome features but not AD. On the other hand, individuals presenting micro-duplicated APP gene but not the whole chromosome 21

develop EOAD but do not get Down syndrome, directly implicating overexpression of APP as a cause of Aβ deposition in adult life [26].

Significant support for the Aβ hypothesis comes from the genetic mutations related to EOAD. Mutations in APP that happen within the Aβ sequence lead to the production of peptides that are characterized by high self-aggregation ability. Moreover, mutations in the genes encoding for active sites of the APP processing enzyme γ-secretase, presenilin 1 (PSEN1) or presenilin2 (PSEN2), alter the proteolytic cleavage of APP, resulting in overproduction of Aβ42 or Aβ43 peptides. These mutations are estimated to be responsible of 71% of EAOD cases, however, that means they explain roughly 0.5 percent of all AD cases given that EAOD accounts for only 1-5% of total AD cases [27, 28]. Astonishingly, inheritance of a missense APP mutation (A673T) located at the second amino acid of the Aβ region results in constant reduction of APP cleavage by β-secretase [29, 30]. Carriers of this mutation show no sign of Amyloid plaques up to 100 years of age, and they have a lower risk of developing clinical AD.

Apolipoprotein E (ApoE) is a lipid-transporter protein involved in the transport and metabolism of cholesterol and other lipids and is immunochemically colocalized with amyloid plaques, neurofibrillary tangles, and vascular amyloid deposits in AD [31]. Due to several single-nucleotide polymorphisms (SNPs), ApoE is found in three isoforms, ε2 (cys112, cys158), ε3 (cys112, arg158), and ε4 (arg112, arg158) with frequencies of 8.4%, 77.9%, and 13.7%, respectively. Despite being the most frequent allele, ε3 is believed to play no significant role in AD, while ε2 is known to be protective and ε4 is shown to be the strongest genetic risk factor in LOAD [31]. Frequency analysis of the three alleles among human populations exhibited robust association between ε4 allele and LOAD with

approximately 40% frequency of the ε4 allele. In a double transgenic mouse model of APP and hApoE, the clearance of Aβ is shown to be decreased by ApoE4 > E3 > E2 in a degree closely related to Aβ deposition in the brain [32]. Therefore, whether it is EOAD or LOAD, genetic risk factors support the amyloid hypothesis, either by promoting the amyloidogenic processing of APP or by disruption to Aβ clearance mechanisms.

While the original amyloid hypotheses (cascade hypothesis) focused more on the senile plaques with respect to their potential toxicity and drug development, the field has shifted towards oligomeric amyloid beta(oAβ) as being the culprit initiating brain damage prior to AD progression. This notion was supported by the clinical observations that amyloid plaque load does not necessarily correlate with the cognitive status of the patients [33]. On the other hand, highly demented individuals carrying a distinct APP mutation, namely the Osaka mutation of APP E693, manifested higher levels of oAβ and other aspects of AD pathology without developing plaques [34-36].

Additionally, several studies showed that soluble oAβ, isolated for human AD brains, led to impairment in synaptic plasticity in the entorhinal cortex and neocortex when injected into the brains of healthy rats[37]. Moreover, human oAβ can trigger tau hyperphosphorylation in primary cultured rat neurons[38].

Considering how intricate AD pathogenesis is, oAβ has been implicated into a rather complex cellular system comprising continuous feedforward and feedback responses between neurons, glial cells, and vasculature. For example, oAβ has been shown to induce reactive oxygen species (ROS) in astrocytes leading to astrogliosis [39, 40]. oAβ has also been found to increase intracellular Ca++ levels in astrocytes within minutes of exposure [40]. Lastly, exposure of both neuron and astrocytes to oAβ leads to activation

of Ca++-sensing receptors, triggering both cell types to secrete nitric oxide (NO), and vascular endothelial growth factor A (VEGF-A), and Aβ42[41]. Astrocytic Aβ has been shown to be secreted encapsulated into extracellular vesicles (like exosomes), possibly contributing to the demise of neurons.

1.1.3 Amyloid beta toxicity in Alzheimer's Disease

As mentioned earlier, Aβ42, in particular oAβ, is widely perceived as the most toxic factor in AD pathogenesis. A well-defined mechanism by which Aβ exerts its toxic effects is not yet clear, however, several groups have proposed potential mechanisms.

One heavily studied mechanism is the effect of Aβ on neuronal synaptic plasticity [42-45]. Synaptic plasticity is an activity-dependent biological process in which neurons brings about changes in the strength and efficacy of synaptic transmission at preexisting synapses, and it is known to play a crucial role in memory and learning [46]. There are two major forms of synaptic plasticity: Long-term potentiation (LTP) which enforces the synaptic transmission, and long-term depression (LTD) which leads to reduction in synaptic function and transmission. α-amino-3-hydroxy5-methyl-isoxazole-4-propinonic acid receptors (AMPARs) and NMDA receptors (NMDARs) are two major ionotropic glutamate receptors that are involved in regulation of synaptic transmission. Regulation of LTP and LTD by both NMDARs and AMPARs involves endocytosis and trafficking of these receptors, that is, when stimulation of NMDR leads to post-synaptic increase in Ca2+ followed by signaling events ending in re-colocalization of new AMPARs to the plasma membrane[47, 48]. In part, Aβ causes synaptic dysfunction by disrupting glutamate levels through aberrant stimulation of NMDARs, which in turn results in desensitization of

NMDARs preventing the induction of LTP. In addition, high levels of Aβ have been associated with increased internalization of AMPAR, which promotes LTD [49-51]. Partial antagonists to NMDARs mitigate the Aβ-induced overstimulation to the receptors in primary neurons of mice and rats. This concept describes the possible mechanism of action of memantine, which is clinically prescribed for AD patients [52, 53]. Moreover, Aβ interacts with the cholinergic receptor α7 nAChR, and promotes NMDA receptors endocytosis and subsequently disrupts cholinergic neurotransmission [54-57].

Aβ has been shown to interact with other receptors as well. Such receptors include Ryanodine receptors, which are calcium-release channels, known to be localized in the soma, spines and proximal dendrites of neurons. Exposure to Aβ leads to enhanced expression and activity of ryanodine receptor 3 and ultimately intracellular Ca 2+ homeostasis disruption [58, 59].

The chemical characteristics of Aβ prompts it to directly interact with lipid membranes in aqueous environments, allowing for interaction with lipids, phospholipids, and membrane-bound proteins[60]. Several groups have reported that Aβ is able to self-assemble within lipid bilayers, creating pore-like hollow structures of annular Aβ oligomers with a hydrophilic interior and hydrophobic exterior[61-64]. This could be due the detergent-like properties of Aβ, allowing it remove lipid molecules and destabilize membranes. Later, this pore-like morphology was confirmed using high-resolution atomic force microscopy and has been found in postmortem brain tissues of AD patients as well[61]. It has been proposed that these Aβ pores work as Ca2++ -sensitive ion channel, which might explain the Ca2++ influx in neurons leading to Ca2++ dyshomeostasis [64].

This in turn might trigger mitochondrial dysfunction instigating a cascade of ROS and subsequently apoptosis[65, 66].

Aβ toxicity has also been traced to intracellular organelles either directly after internalization or as a consequence of initial cell surface interaction, one of these organelles are mitochondria[67-69]. In fact, a decrease in glucose metabolism was observed in areas associated with amyloid plaques, implicating Aβ in the process[70]. In addition, mitochondrial dysfunction was observed prior to the amyloid plaque deposition[71, 72].

As mentioned earlier, Aβ can penetrate lipid membranes, including mitochondrial membranes, and has been reported to disrupt the electron transport chains [73-76]. Aβ decreases the activity of the terminal enzyme in the electron transport chain cytochrome C, by interfering with its binding to cytochrome c oxidase [67, 77-80]. Another enzyme that is shown to be affected by Aβ is amyloid-binding alcohol dehydrogenase (ABAD), which is a multifunctional mitochondrial enzyme involved in energy production and is upregulated in AD [81, 82]. ABAD undergoes conformational changes when bound to Aβ, which prevents the enzyme from binding to NAD+, resulting in the loss of ABAD enzymatic activity [81].

Another form of the deleterious effect of Aβ on mitochondria is the direct dysregulation of mitochondrial fission and fusion [83, 84]. As the terms suggest, mitochondrial fusion means combining two mitochondria into one, while fission means dividing one into two [85, 86]. Fusion and fission are controlled dynamic processes that occur at a high frequency in response to cellular and mitochondrial conditions. Dysregulation of fission and fusion leads to either elongated misshapen mitochondria

(excessive fusion) or mitochondrial fragmentation (disproportionate fission). One key regulator of mitochondrial fission is the dynamin-like GTPase protein dynamin-related protein 1 (DRP1) [87-89]. It has been shown that Aβ activates DRP1 through S-nitrosylation causing mitochondrial fragmentation and synaptic degeneration in AD [90].

Voltage-dependent anion channel 1 (VDAC1) also interacts with Aβ monomers and oligomers[91-93], and the block of those mitochondrial pores leads to mitochondrial dysfunction. VDAC1 is a mitochondrial gatekeeper located on the outer mitochondrial membrane that regulates the metabolic cross-talk between mitochondria and the rest of the cell[94, 95]. VDAC1 regulates the entry of metabolites into the mitochondria including malate, nucleotide, succinate, and NADH while allowing the release of hemes and other molecules outside of mitochondria. Under normal physiological conditions, VDAC1 is found in a dynamic equilibrium between monomeric and dimeric states. However, when VDAC1 is subjected to an insult, it assembles into a higher oligomeric structure, forming a channel. Those channels allow the passage of cytochrome C to the cytosol, ultimately leading to apoptosis[96-98].

Recent evidence has implicated the VDAC1 in AD pathogenesis, evident by the demonstration that interactions between VDAC1, Aβ, and phosphorylated tau were shown to lead to mitochondrial dysfunction[99, 100]. In addition, Hippocampal extracts of AD transgenic mice showed increased levels of VDAC1 in the outer mitochondrial membranes. Higher expression levels of VDAC1 were demonstrated in the dystrophic neurites with Aβ deposits in the brains of both AβPP transgenic mice and AD post-mortem individuals, as well[101]. Lastly, soluble oligomeric Aβ is reported to cause apoptosis in neurons as a result of upregulation and oligomerization of VDAC1[102].

1.2 Extracellular vesicles

1.2.1 History and Background

The phenomenon of cellular secretion of small vesicles was first reported in the late 1960s, when Bonucci and Anderson noticed that chondrocytes secrete nanosized vesicles of ~100 nm[103, 104]. During the same time period, another group showed that platelets secrete small vesicles as well, which they referred to as platelet dust[105]. These extracellular vesicles (EVs) were overlooked for two decades, but were then discovered to harbor cell specific proteins, genetic material and lipids that are delivered to other cells where they could alter diverse functions[106-109].

The first reported functional role of EVs came from the field of immunology. B lymphocytes secreted EVs and demonstrated a transfer of antigens, major histocompatibility peptide complexes that are recognized by T lymphocytes, suggesting a role of EVs in immune response induction[110]. Two years later, the same group demonstrated that dendritic cells secrete EVs bearing functional peptide complexes as well, which can trigger antitumor immune responses in mice, *in vivo* [111]. These results provided the groundwork for the popular concept that EVs could play an active role in intercellular communication, at least in the immune system. This hypothesis proved to hold true in several other systems as well. Another breakthrough took place in 2007 when Jan Lötvall and group members described the presence of microRNA (miRNA) inside these vesicles, leading to a surge in the number of studies and publications in the pursuing years[112].

The term “exosomes’ describes the intra luminal vesicles (ILVs) that originate in the multivesicular bodies (MVBs). This terminology is widely accepted; however, the word “exosomes” was not actually implemented until 1987[113]. Microvesicles are another well-studied, secreted vesicle[114, 115]. One notable difference between the two types of

EVs is the size. While exosomes, or small extra cellular vesicles, have a size range of ~40 to 160 nm (with an average of ~100 nm) in diameter, the size of microvesicles usually is in the range of ~150-1000nm in diameter[116, 117]. This difference allows for the separation of the two types of vesicles via differential ultracentrifugation. In most cases, larger vesicles preferentially sediment at a relatively low g force (e.g., 30 min at 10,000 × g), while smaller vesicles need a higher g force (e.g., 90–120 min at 70,000–120,000 × g) to sediment.

Another apparent difference between exosomes and microvesicles is that exosomes are specifically enriched with certain types of cell-specific proteins, lipids, and nucleic acids[118]. On the other hand, microvesicles are a smaller representation of the cells of origin, in terms of composition. Granted, there are other types of microvesicles reported in literature, such as apoptotic bodies and ectosomes, which are derived from cells undergoing apoptosis and plasma membrane shedding, respectively[119, 120]. Although apoptotic bodies, ectosomes and exosomes roughly share same size (typically 40–100 nm) they are essentially different species of vesicles.

1.2.2 Biogenesis

Exosomes are typically formed through a process that involves double invagination of the plasma membrane leading to the formation of multivesicular bodies (MVBs) that contain intraluminal vesicles (ILVs)[113]. This process was first observed during studies concerning elimination of transferrin receptor (TfR) from the plasma membrane of maturing reticulocytes[121]. The process of MVB biogenesis was shown to involve selective endocytosis of TfR from the plasma membrane, followed by budding of TfR from the endosomes' membrane into the endosomal lumen, and then fusion of the MVBs with the plasma membrane. There are several routes that MVBs could take. While some MVBs fuse to the plasma membrane to release there ILVs (exosomes) into the extracellular milieu,

others fuse with lysosomes or autophagosomes for degradations, or contribute to specialized organelles generation such as Weibel-Palade bodies (endothelial cells), secretory granules (in mast cells), and melanosomes (in melanocytes)[122]. It is thought that the cholesterol level within MVBs plays a role it their fate, while cholesterol-rich MVBs are directed to the plasma membrane, alternatively, the cholesterol-poor MVBs favor the lysosome fusion fate[123].

In general, MVB biogenesis is driven by either endosomal sorting complexes required for transport machinery (ESCRT)-dependent and or ESCRT-independent pathways (Fig. 1.2)[124]. The ESCRT machinery consists of a distinct group of cytosolic protein complexes (ESCRT-0, -I, -II and -III). These complexes orchestrate the ILVs generation in a stepwise fashion. They are recruited to endosomes through interaction of ESCRT-0 and ESCRT-I with tagged, usually ubiquitylated, transmembrane proteins from microdomains in the endosomal membrane followed by recruitment of ESCRT- III. ESCRT-II is the last complex to engage in this process, and it is believed to be responsible for vesicles detachment and release.

The ESCRT machinery participates in exosome biogenesis through other mechanisms as well, one of which involves the syndecan-syntenin-ALIX axis as recently described by Baietti et al[125]. They report that the syndecan heparan sulphate proteoglycans, with the help of their cytoplasmic adaptor syntenin, regulate endosomal biogenesis of exosomes. ALIX protein is known to be functionally important for the ESCRT machinery through interacting with several ESCRT proteins, like TSG101 and CHMP4. In their study, Baietti et al. showed that Syntenin binds directly to ALIX and triggers the intraluminal abscission of endosomal membranes.

Several lines of evidence indicate an overlap between the autophagy pathway and exosome biogenesis and secretion[126-128]. This was predictable due to the verity that autophagy is a lysosomal-dependent degradation and recycling pathway, and it is plausible that autophagy shares some molecular machinery with exosomes biogenesis. In fact, it has

been recently shown that autophagy pathways secrete waste in endosomal-derived vesicles, resembling the exosome biogenesis process. Many proteins have been identified as key players in both autophagy and exosome production. A recent study by Gudbergsson and Johnsen compared a list of proteins involved in several autophagy pathways to that of EV protein database named Vesiclepedia[129]. The authors reported that nearly all autophagy key proteins have been identified in EV literature[130]. Noticeably, four of the autophagy-related proteins were HSPA8, HSP90AA1, VCP, and Rab7A all of which are well recognized proteins in EV production processes.

Another shared protein between autophagy and exosome biogenesis is the class III PI3K complex. This complex includes proteins such as Beclin-1, p150, VPS34, and other accessory proteins. PI3k exerts its regulatory role through the generation of PI (3)P via phosphatidyl inositide phosphorylation[131]. With the help of other proteins, namely ATG14L and UVRAG, PI (3)P regulates autophagosome maturation and endosome development, respectively[132].

The extensive mention of MVBs as the origin of exosomes in literature does not necessarily mean that endosomal budding is the only method for the generation of exosomes. Stephen J. Gould and others have shown in several reports that exosomes can be formed through direct budding from the plasma membrane[133-135]. Unfortunately, these are largely ignored by most models of exosome biogenesis, which conveys an endosome-only view of exosome biogenesis. This relatively new mechanism has been observed using techniques such as atomic force microscopy, electron microscopy, and cryo-electron microscopy. In addition, recent studies show the presence of deep invagination in the plasma membrane, that resembles MVBs, when using conventional transmission electron microscopy. These invaginations are called intracellular plasma membrane–connected compartments (IPMCs) and are shown to be continuous with the extracellular space through necks, allowing free passage of small-molecules and extracellular buffer while preventing the escape of vesicles. This process might explain the

enrichment of integral membrane proteins or proteins within lipid rafts of the plasma membrane and in the newly formed exosomes.

1.2.3 Sphingolipids in Extracellular vesicle

In ESCRT-independent manner, two enzymes involved in lipid metabolism have been shown to drive the formation of ILVs in in the lumen of MVBs. Phospholipase D2(PLD2) hydrolyzes phosphatidylcholine into phosphatidic acid (PA) at the inner leaflet of late endosome membranes[136]. The negative charge of PA drives the inward budding of ILVs inside MVBs. The second enzyme involved in ESCRT-independent exosomes biogenesis is neutral sphingomyelinase 2(nSMase2)[137]. nSMase2 hydrolyze sphingomyelin to produce ceramide[138], a cone-shaped sphingolipid that triggers spontaneous curvature of membranes leading to invagination and budding of exosomes into the late endosomes (MVBs).

Sphingolipids constitute a major component of membranes in eukaryotic cells[139]. The first sphingolipids were isolated from the brain in the late 19th century by Thudichum[140]. He then coined the term “sphingosin” after the Greek mythical creature, Sphinx, as they presented as enigmatic molecules. Sphingolipids are a very complex and diverse group of lipids comprised of hundreds of lipids[141-143]. In addition to being a part of cell membrane, sphingolipids are shown to be involved in the formation of structures called” lipid rafts”, roughly 50-200nm diameter microdomains that are enriched with certain sphingolipids and incorporate proteins, named raft-associated proteins (RAPs)[144-146]. The presence of RAPs within the lipid rafts implicates these microdomains in cell signaling integration. Ceramide is the core constituent of all complex sphingolipids and its structure consists of a sphingosine long-chain backbone linked to a fatty acid via an amide linkage[139, 147].

In addition to exosome biogenesis, ceramide is a key player in various aspects of cellular processes including growth, survival, senescence, proliferation, and apoptosis. This functionality of ceramide qualifies it as a biologically active cell signaling lipid, along with other sphingolipids such as sphingosine 1 phosphate(S1P) and sphingosine [145].

Ceramide synthesis can occur via two distinct pathways; the anabolic (also known as de novo) and the catabolic (frequently referred to as salvage) pathways [148, 149] (Fig. 1.3). The de novo pathway is initiated by the condensation of serine and palmitoyl-CoA to produce 3-ketodihydrosphinganine, catalyzed by the enzyme serine palmitoyl transferase (SPT). 3-ketodihydrosphinganine is then reduced to sphinganine (Dihydrosphingosine) through the enzymatic action of enzyme 3-ketodihydrosphingosine reductase. Sphinganine is N-acylated to dihydroceramides (DHCer) by ceramide synthases (CerS), a family of acyl-CoA transferases that controls the fatty acyl chain length. There are six established CerSs in mammalian cells, with CerS1 being the most abundant one in the brain(neurons), specifically attaching C18 fatty acyl CoA to the sphingoid base. Finally, ceramide formation is completed by the enzyme dihydroceramide desaturase through desaturation of the of DHCer, which introduces a 4,5-trans double bond at the sphinganine base of DHCer[138, 139].

On the other hand, the salvage pathway re-utilizes long chain sphingoid bases, mostly from sphingomyelin, to produce ceramide. Sphingomyelin represents the most abundant sphingolipid in cell membranes, subsequently playing a vital role in membrane fluidity and homeostasis. Sphingomyelin is a substrate for a family of enzymes called sphingomyelinases (SMases) which hydrolyze sphingomyelin into ceramide and phosphocholine. Five SMases have been identified in literature and they can be generally separated in to two groups, namely acid and neutral SMases, depending on their subcellular localization, pH optima, and cation requirements for enzymatic activity. It has been speculated that nSMase2, the brain specific form of SMases, is present in an inactive form under normal conditions. Upon stimulation with different extracellular factors and

intracellular processes, including tumor necrosis factor α (TNF-α), interleukin 1β/6, and oxidative stress, nSMase2 then moves to the plasma membrane where it facilitates ceramide production and the downstream effects[150]. In addition, nSMase2 has been shown to partake in pro-inflammatory cytokine signaling, implicating the enzyme in the progression of several neurodegenerative diseases[151, 152].

In general, alteration in ceramide (and other sphingolipids) levels or metabolism has been linked to numerous neurodegenerative diseases including epilepsy, Parkinson's disease, Huntington's disease, Gaucher's disease, Krabbe's disease, and AD[153-158]. Ceramide has been proven to have a multifaceted role in neurodegenerative diseases, especially AD. Due to advances in mass spectrometric methods, researchers were able to show that ceramide levels are elevated in the serum, cerebrospinal fluid (CSF), and brains tissues of AD patients[159-162]. In addition, plasma ceramide levels have been shown to directly correlate with brain hippocampal volume in late onset AD patients.

As a part of The Women's Health and Aging Study ‖, Mielke and colleagues followed 99 cognitively normal older women for nine years in a prospective cohort study. They were able to show concrete relationship between higher baseline serum ceramide levels and the risk of developing dementia and AD, priming serum ceramide as a biomarker for preclinical AD. More specifically, researchers were able to narrow down certain ceramide species that are elevated in aging and AD, such as Cer16, Cer18, Cer20, Cer24, and Cer24:1[161].

On a cellular level, endogenous ceramide within lipid rafts in the plasma membrane, as well as the ceramide analog C6-ceramide, were shown to directly increase the production of Aβ by stabilizing the β-site APP cleaving enzymes[163]. Moreover, exposure to Aβ activates nSMase 2 *in vitro*, which might be associated with facilitating Aβ aggregation and spreading given the role of nSMase 2/ceramide in exosomes production[164].

Concomitantly, Aβ can indirectly increase the production of ceramide through an oxidative stress-mediated mechanism[165, 166]. Exosomes formed through the nSMase 2 pathway are being packaged with misfolded proteins, which might provide an efficient way for cellular uptake during different neurodegenerative diseases[167]. In fact, oligomeric α-synuclein displayed higher levels of cellular uptake when associated with exosomes rather than its free form[168].

It is worth mentioning that all the previous reports pertaining the interplay between ceramide and Aβ have been conducted in neuronal cells. Recently, the same concept has been gathering interest in glial cell. First, ceramide has been shown to sensitize astrocytes to oxidative stress[169]. Human post-mortem tissues of frontotemporal lobar dementia (FTLD) demonstrated increased levels of ceramide in astrocytes as well, which correlated with the level of neuroinflammation[170]. In addition, treating primary astrocytes with oligomeric Aβ led to the release of ceramide enriched exosomes that cause apoptosis to recipient astrocytes[164].

The presence of ceramide in several cellular membranes leads to the discovery of its role as a regulator of organelle functions and cellular transport. This role involves regulation of membrane curvature, fission and fusion, endocytosis, cellular transport, vesicular transport, as well as mitochondrial function[171-175]. Ceramide regulation of mitochondrial function is multifaceted, it could assist in protein transfer, interact with mitochondrial membrane proteins, or directly interact with the membrane lipid bilayer[176-179]. Excess ceramide that dysregulates mitochondrial function may originate from the upregulation of endogenous ceramide generation, from the endoplasmic reticulum, other organelles or the uptake of ceramide contained within “mobile lipid rafts” in exosomes. Ceramide has been reported to increase the mitochondrial outer membrane permeability through interactions with several kinases and phosphatases that govern the apoptosis process. For instance, elevated ceramide levels inhibit phosphoinositide-3-kinase (PI3K) and Akt/PBK signaling, which results in the activation of pro-apoptotic Bcl-2-

family protein Bad[180]. In addition, coarse-grain molecular dynamics simulations showed that VDAC1 carries a ceramide binding site, directly implicating ceramide in mitochondrial apoptosis[181]. In model bilayers and isolated mitochondrial outer membranes, ceramide was found to form pores large enough to that allows the passage of cytochrome c[182].

1.2.4 Exosomes in AD

Exosomes were first implicated in AD as carriers of pathogenic proteins and causative of impaired neuronal function[183, 184]. Firstly, Aβ peptides were shown to accumulate in MVBs using electron microscopy on AD transgenic mouse models as well as human AD brain sections[185]. This was followed up a study showing colocalization between flotillin-1, the raft and exosomal marker, with Aβ in the lumen of MVBs.

In 2006, Rajendran and colleagues were first to show that β-cleavage of APP occurs in early endosomes[186]. That is followed by routing Aβ to MVBs in N2a and Hela cells. The same study estimated that a minute fraction of Aβ (<1%) is then secreted from cells to the extracellular space in association with exosomes. They also found that the ESCRT accessory protein Alix to accumulate in the plaques of AD patient brains, suggesting a role of exosomes in Aβ aggregation and plaque formation. Further evidence for the role of MVBs in APP metabolism came from the study on SY5Y neuroblastoma cells. Upon treatment of SY5Y cells with alkalizing drugs like chloroquine or bafilomycin A1, APP CTFs and amyloid intracellular domain (AICD) accumulate in MVBs prior to their secretion within exosomes[187]. Recently, the packaging of APP into exosomes has

been shown to be regulated by vesicle-associated proteins Alix and Syntenin-1[188]. Furthermore, APP-processing enzymes (β-secretase and γ-secretase components PS1 and presenilin-2) were shown to be secreted in exosomes, using Chinese hamster ovary (CHO) cells[189]. Same exosomes contained APP-CTFs and Aβ. More recently, a group in Sweden showed that intact exosomes and their cargo, including human AD brain oAβ, can be transferred from one neuron to another using a co-culture method that utilizes two different neuronal cell types[190]. The authors provided evidence that exosome uptake into neurons is an active process (dynamin dependent) and preincubation of cells with the endocytosis inhibitor dynasore leads to significant reduction in oAβ uptake, propagation, and subsequent toxicity. The previous studies not only implicate exosomes in spreading of Aβ, but also in facilitating APP cleavage into Aβ in the recipient neurons.

Tau, on the other hand, is known to spread in AD brain in a well-defined manner, from one region to another, with the uptake of pathological tau causing misfolded aggregations of monomeric tau in recipient cells[191-193]. This “prion like mechanism” of spreading led scientists to propose a role of exosomes in the propagation of tau. Indeed, tau phosphorylated at Thr-181(AT270) was found associated with exosomes in human CSF as well as M1C neuroblastoma cell cultures[194]. Notably, exosome-associated tau (relative to free tau) is elevated in CSF during mild AD, suggesting a role of exosomal tau secretion in the abnormal processing of tau, preceding cell death at later stages. The same authors took this study further by demonstrating that overexpression of human tau in neuroblastoma cells leads to recruiting mitochondrial proteins that are involved in axonogenesis relevant to neurodegeneration into the exosomal secretion pathway[195]. In

another overexpression study, tau was found to be secreted in association with membrane vesicles after reaching a threshold intracellular concentration level[196, 197].

Asai and colleagues generated the most compelling study by demonstrating the role of exosomes in tau propagation[198]. Their *in vivo* study was the first to display the rather surprising role of microglial exosomes as tau spreads. Using two separate mouse models, the authors were able to show that microglia phagocytose tau was followed by exocytosis in association with exosomes. They continued to demonstrate that depletion of microglia through either i.c.v. infused clodronate liposomes or feeding the mice an inhibitor of colony stimulating factor 1 receptor (CSF1R) resulted in significant reduction in tau propagation. They also showed that inhibition of exosome synthesis significantly reduced tau propagation *in vitro* and *in vivo*. The implication of microglial EVs in spreading of toxic pathogens is further supported by a study showing that treating primary microglia with Aβ leads to the production of Aβ-containing macrovesicles that are *neurotoxic in vitro* [199]. Recently, one study linked Bridging Integrator 1 (BIN1), a major locus associated with LOAD, to the secretion of tau in exosomes [200]. It was reported that overexpression of BIN1 triggered the release of tau-associated exosomes and genetic deletion of the gene from microglia significantly reduced tau spreading *in vivo*.

Apart from being a carrier for several pathogens involved in AD and other neurodegenerative diseases, exosomes have also been shown to have neurotoxic effects relative to AD pathology. For instance, primary astrocytes treated with Aβ were shown to secrete exosomes that carry Aβ, enriched with ceramide, and harbor the pro-apoptosis prostate apoptosis response-4(PAR-4)[164]. These exosomes proved to induce apoptosis in recipient astrocytes. More recently, Ikezu's group reported that activated human

astrocytes (treated with interleukin-1β) secrete exosomes with higher level of enrichment of integrins and major histocompatibility complex[201]. These exosomes lead to neurite fragmentation accompanied by a reduction in neuronal branching and firing, when they were added to primary cultured mouse cortical neurons. On subcellular level, exosomes from the CSF of late-onset AD or PS1 mutation-harboring cells were shown to impair Ca2+ handling and mitochondrial function in cultured neurons[202].

Despite the plethora of articles attributing toxic effect to exosomes, other beneficial roles of exosomes have also been reported. The initial reports pertaining to the advantageous role of exosomes in AD came in a series of publications from Yuyama and colleagues, describing an exosome dependent Aβ clearance[203]. The authors utilized a transwell co-culture system of human APP-transfected N2a cells and microglial BV2 cells to show that microglia engulf neuronal exosomes containing Aβ, which participates in the clearance of the peptide and reducing its pathology. They followed up with an *in vivo* study two years later where they continuously infused neuronal exosomes intracranially into transgenic AD mouse brains for two weeks using osmotic pumps[204]. They were able to demonstrate that exosomes injections decreased the levels of Aβ and ameliorated its toxicity with respect to synaptic density reduction in hippocampus of four month-old mice. They reported similar findings in 12 months old transgenic mice, as well. Of note, the exosomes used in these studies came from normal wild type neurons or neuroblastoma cell lines and the exact mechanism of the protective role of these exosomes remains elusive. Another example of the beneficial role of exosomes in AD came from a separate *in vivo* study using Intracerebroventricularly (i.c.v) infusion of N2a cells or human cerebrospinal fluid derived exosomes. These exosomes were shown to abrogate the synaptotoxic effects

(disruption of long-term potentiation) of both synthetic and AD brain-derived Aβ. In this study, Aβ peptides were shown to be sequestered by exosomes with the help of exosomal surface proteins including cellular prion protein (PrPC)[205]. Additionally, human umbilical cord mesenchymal stem cells (huc-MSC)-derived exosomes proved to exhibit preventive effects when injected into double transgenic mouse model of AD(APP/PS1)[206]. These exosomes helped abrogating neuroinflammation through altering the levels of expression of pro- and anti-inflammatory cytokines. The effect of these exosomes extended to enhance spatial learning and memory function in addition to reducing the amyloid plaques load in the cortex and hippocampus on the injected animals.

Since exosomes are capable of carrying proteins as cargo, it is not surprising that several groups reported the presence of Aβ degrading enzymes in exosomes, representing another potential protective role of exosomes in AD[207-210]. Some of the enzymes reported were found in the last-mentioned study since the authors reported upregulated levels of insulin-degrading enzyme (IDE) and neprilysin in the exosomes. Others reported the presence of metalloproteinases and endothelin-converting enzymes, both have Aβ-degrading capabilities, in association with exosomes.

Being biological entities, exosomes are known to freely cross the blood brain barrier (BBB) in both directions[211-213]. This unique capability posed exosomes as an exciting drug delivery method. In addition, the presence of exosomes in peripheral circulation allowed researchers to capture them, away from the brain, and study their content and cargo aiming to find unique exosomal biomarkers relative to neurodegenerative diseases, making them a potential diagnostic tool.

Exosomes contain constituents of their cells of origin, which turns them into biomarkers for the secreting cells. Utilizing this concept, efforts have been made to enrich for exosomes from distinct brain cells, especially neurons and astrocytes. In the context of AD, it does not come to surprise that the first thought after targets were the major culprits in AD pathophysiology, namely Aβ, tau, and p-tau. Precisely the Aβ1-42/1-40 ratio, T -tau and p -tau are being already used as biomarkers in CSF-based neurochemical diagnosis, and the same molecules have been identified in serum exosomes[214, 215]. Fiandaca and colleagues reported that neuronal exosomes enriched from the blood of AD patients showed significantly higher levels of Aβ1-42, total tau, p-tau181, and p-tau 396 compared to the controls[215]. The levels of these candidates were higher in preclinical individuals up to ten years prior to the clinical onset of AD. Similar results were reported by another group, claiming that plasma exosomes from AD patient significantly differed from healthy control based on their morphology, content, and count which might provide a basis for early diagnosis of AD[216]. In a recent elegant study, a new technology termed amplified plasmonic exosome (APEX) was developed to Subtype blood exosome-bound Aβ in relation to cells of origin. The authors found that plasma neuronal exosomes favor binding to prefibrillar Aβ, specially Aβ1-42. Analyzing these exosomes with APEX presented a precise correlation to the amyloid plaque load in AD patients with higher sensitivity than positron emission topography (PET), especially in early stages of AD[217].

Although Astrocyte-derived exosomes (ADE) are reported to be present at lower levels in plasma compared to neuronal ones (NDE), they contain a higher amounts of APP-derived metabolites (including Aβ1-42) and APP-processing enzymes like BACE-1 in addition to p-tau[218]. This observation allows ADE to distinguish AD patients from the

study controls. Recently, the components of the complement system have been identified in plasma astrocytes-derived exosomes, implicating ADE in inflammation and neurodegeneration in AD[219, 220]

1.3 5xFAD transgenic mouse model

In this work, we use the AD mouse model called 5xFAD. This terminology stems from the fact that these mice express a total of five AD related mutations. Three of the mutations are human APP which are the Florida (I716V), Swedish (K670N/M671L), and London (V717I) while the other two are pertaining PSEN1, namely M146L and L286V. These genes are regulated by the neural-specific Thy1 promoter, which might explain why females produce more Aβ than males, probably due to an estrogen response element in the Thy1 promoter used to drive transgene expression. 5xFAD mice recapitulate the AD phenotype in several aspects. Intraneural Aβ begins to appear as early as 1.5 months of age while senile plaques are observed at two months of age[221]. BACE1 increases in these mice in an age-dependent manner, as well. Gliosis has been reported to accompany plaque deposition in two month old mice. Senile plaques are observed firstly in the subiculum and layer V of the cortex, pathology then appears in the cortex and hippocampus by six months of age[221]. Older mice show plaques in the brainstem, thalamus, and olfactory bulb. By four months, synaptic degeneration commences, which is determined by the decrease in presynaptic markers synaptophysin and syntaxin throughout the entire brain. Post synaptic markers begin to decline by nine months. Lastly, multiple regions of brain exhibit neuronal loss which starts in areas with severe plaque depositions at about six months of age.

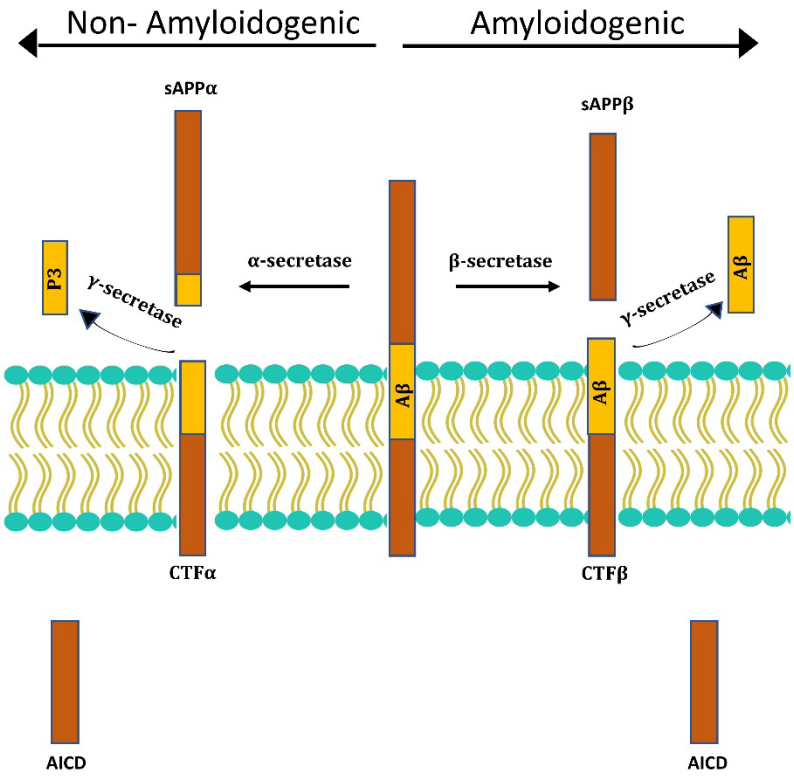

Figure1.1: Overview of APP processing. In the non-amyloidogenic pathway, α-secretase cleaves APP creating sAPPα and CTFα, which is subsequently cleaved by γ-secretase into P3 and AICD. In the amyloidogenic pathway, APP is first processed by β-secretase, where sAPPβ is released, followed by cleavage of the CTFβ by γ-secretase to release Aβ.

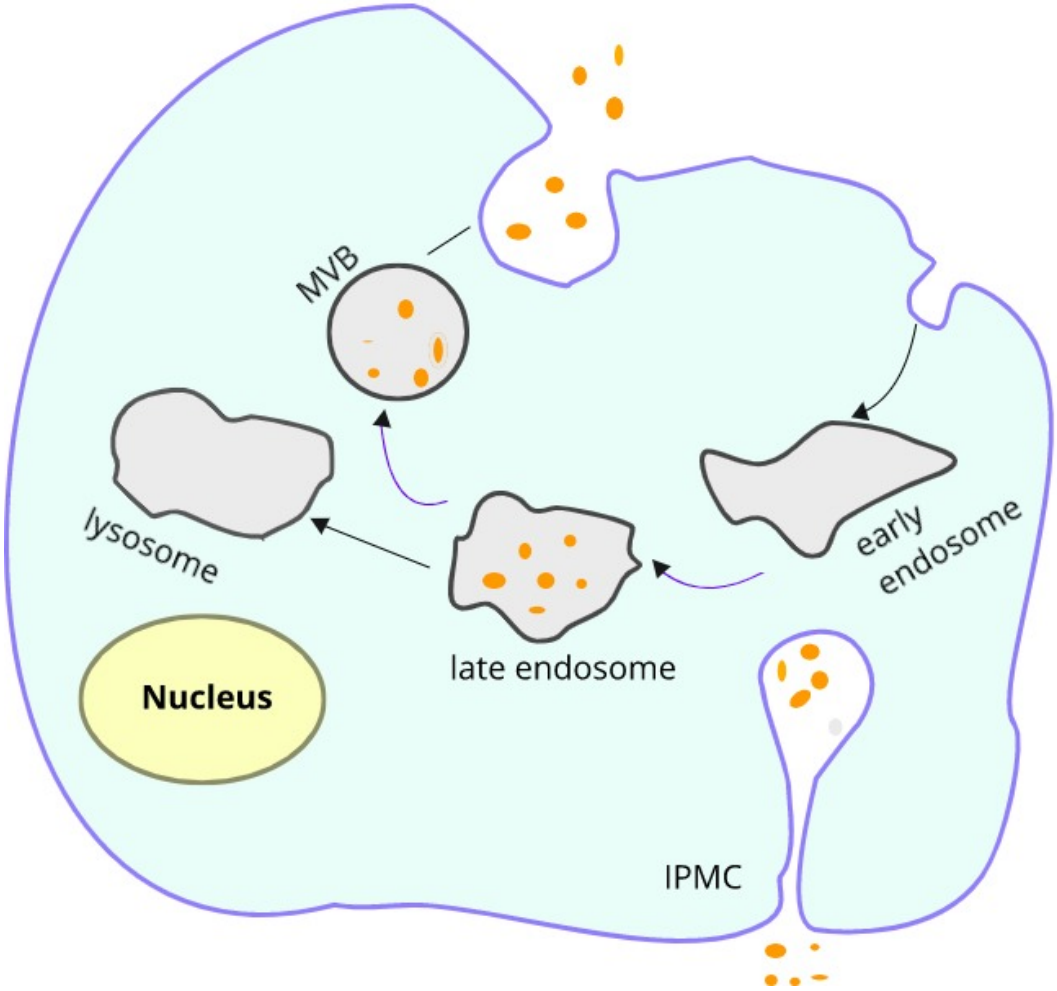

Figure 1.2: Overview of exosome biogenesis mechanisms involving MVBs and IMPCs. Early endosomes mature into multivesicular bodies (MVBs), which are late endocytic compartments containing intraluminal vesicles (ILVs). Fusion of MVBs with the plasma membrane results in the release of ILVs into the extracellular space as exosomes. Other vesicles can be shed from the cell by direct budding from the plasma membrane (e.g. microvesicles and IPMC derived vesicles). Multiple machineries are involved in the biogenesis of ILVs, and thus of exosomes, such as ESCRT components, tetraspanins, and lipids.

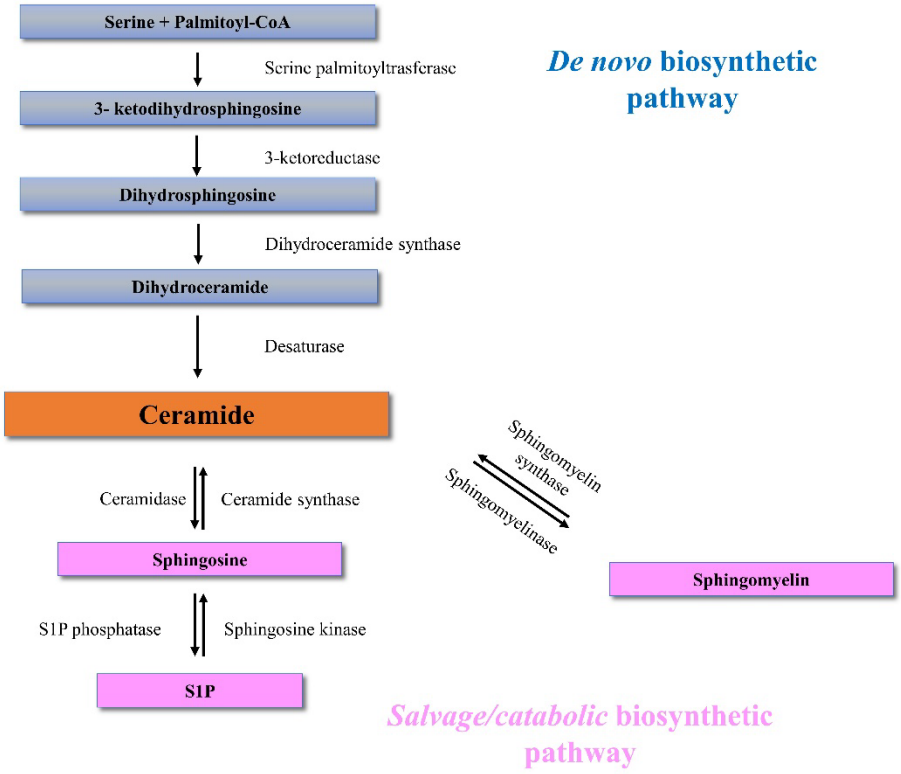

Figure 1.3: Overview of sphingolipid metabolism and related enzymes.
The *de novo* biosynthetic pathway is initiated in the endoplasmic reticulum by the action of serine palmitoyltransferase (SPT). Generation of ceramides happens after several enzymatic reactions. Ceramides are then incorporated into various complex sphingolipids (predominantly in the Golgi) including ceramide-1-phosphate (C1P), sphingomyelin or glycoceramides. In sphingolipid catabolic pathways, mostly in lysosomes, sphingomyelin (also glycosphingolipids and ceramide-1-phosphate) are hydrolyzed, resulting in the formation of ceramide. Ceramide can then be deacylated to generate sphingosine, which in turn is phosphorylated to sphingosine-1-phosphate (S1P).

CHAPTER 2. ASSOCIATION OF AB WITH CERAMIDE-ENRICHED ASTROSOMES MEDIATES AB NEUROTOXICITY

2.1 Introduction

Aβ plaque deposits and tau neurofibrillary tangle formation are hallmarks of AD [222, 223]. However, it is still controversial which of the two factors is critical for neuronal dysfunction and death, ultimately leading to cognitive decline and demise of the patient. Most of the previous studies assumed that the buildup of Aβ or tau by themselves induces neurotoxicity[44, 224, 225] . This assumption, however, was in stark contrast to observations in AD mouse models and patients showing significant buildup of plaques and tangles without obvious neuronal cell death[223]. We hypothesized that neuro- toxicity of Aβ is mediated by its interaction with an unknown factor. Based on our previous studies showing that Aβ associates with astrocyte-derived exosomes (here termed astrosomes), we tested if this interaction mediates neurotoxicity of Aβ[164, 226].

Exosomes are generated as intraluminal vesicles of multivesicular endosomes and secreted as a type of extracellular vesicles by a large variety of cells and tissues[106, 227, 228]. Exosomes are deemed to serve as carriers for the intercellular transport of micro RNAs and some proteins. Although their size of 100 nm favors a high membrane surface-to-volume ratio, the role of membrane lipids in exosomes remains largely un-explored[229, 230]. Our laboratory discovered that the sphingolipid ceramide is enriched in the membrane of astrosomes[164]. We also showed that ceramide mediates association of Aβ with astrosomes and that this association leads to astrosome aggregation *in vitro*, a process we suggested to nucleate amyloid plaques in AD brain [226]. However, we do not know if amyloid plaque nucleation is the only or even main function of astrosomes. Recent studies demonstrated that Aβ-associated exosomes cross the blood-brain-barrier and are

detectable in serum from AD mice and patients[189, 215, 231]. In fact, exosomes purified from patient serum are proposed as AD biomarkers that are detectable up to a decade prior to clinical symptoms of cognitive decline[215]. While a proportion of serum exosomes is clearly derived from brain, composition and function of these exosomes remains largely unknown.

In the current study using mass spectrometry and anti-ceramide antibody, we found that a proportion of serum-derived serum exosomes is enriched with the same ceramide species previously detected in astrosomes isolated from primary astrocyte culture[226]. We also isolated exosomes from wild type and 5xFAD brain tissue and confirmed the astrocytic origin and Aβ association of tissue and serum-derived exosomes by testing for the presence of the astrocyte marker glial fibrillary acidic protein (GFAP) and Aβ. Aβ-associated astrosomes were taken up by neural cells and specifically transported to mitochondria, thereby inducing mitochondrial damage and caspase activation. Most importantly, the concentration of Aβ associated with astrosomes inducing damage was several orders of magnitude lower than required when using Aβ without astrosomes. Aβ-associated astrosomes induced formation of a pro- apoptotic complex between Aβ and voltage-dependent anion channel 1 (VDAC1), the main ADP/ATP transporter in the outer mitochondrial membrane[232, 233]. These results suggest that astrosomes are the unknown factor mediating neurotoxicity of Aβ by inducing mitochondrial damage and apoptosis. Our data also indicate that Aβ-associated exosomes may comprise a novel pharmacological target for AD therapy.

2.2 Methods

Cell cultures: The N2a cell line was obtained from ATCC (CCL-131™). The cells were grown to 90% confluence at 37 °C and 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS) on 100 mm plates (Corning, MA, USA). For immunocytochemistry analyses, cells were seeded on poly-L-lysine (Milipore- Sigma, Montana, USA) coated cover slips at 10,000 cells/cover slip. Cells were gradually deprived of serum to allow for differentiation into neuron-like cells. Incubation with exosomes was always performed under serum-free conditions.

Primary neurons were isolated from E16.5-P0 mouse cortices following 30 min trypsinization and trituration with a flame-polished Pasteur pipet. Neurons were plated on polyethylene imine coated T-25 flasks as previously described[226] and maintained 7 days in Neurobasal medium with B27 supplement (Life Technologies) prior to incubation with exosomes. To cultivate human induced pluripotent stem (iPS) cell-derived neuroprogenitor (NP) cells, the ReNcell VM Human NP cell line was obtained from Millipore (Temecula, CA, USA, Cat# SCC008). Cells were maintained according to the supplier's protocol. Briefly, cells were expanded on laminin- coated 100 mm tissue culture dishes (Corning) in ReNcell NSC maintenance medium (Millipore) supplemented with 20 ng/mL fibroblast growth factor–2 (FGF-2) and 20 ng/mL epidermal growth factor (EGF) (Milli- pore). The medium was changed daily during the maintenance period. The cells were passaged once a week using Accutase (Millipore). Cells were then differentiated by seeding them at around 60% confluency on freshly laminin-coated dishes and growing overnight in the

presence of growth factors, followed by withdrawal of growth factors. The media were replaced every other day up to 10 days during the differentiation period.

Serum exosome isolation, quantification, and labeling: All experiments using mice were carried out according to an Animal Use Protocol approved by the Institutional Animal Care and Use Committee at University of Kentucky. Sera were isolated from freshly obtained mouse blood. Human exosomes were isolated from sera obtained from the University of Kentucky Alzheimer Disease Center.

Mouse blood was drawn through heart puncture and was allowed to clot at room temperature for 30 min. Blood was then centrifuged at 1800 x g for 10 min at 4 °C. The clear upper layer was transferred to a fresh tube and centrifuged at 3000 x g for 15 min to pellet residual blood cells. Exosomes were extracted using ExoQuick exosome solution (EXOQ; System Biosciences, Inc., Mountain View, CA, USA) according to the manufacturer's protocol. Briefly, 250 µl aliquots of serum were treated with 67 µl of ExoQuick exosome solution, followed by incubation for 60 min at 4 °C to precipitate total exosomes. Tubes were then centrifuged at 1500 x g for 30 min. Each exosome pellet was resuspended in 100 µl of PBS with 1X Halt™ Protease Inhibitor Cocktail (Thermo Fisher, Massachusetts, USA). In certain experiments exosomes were labeled with PKH67 Green Fluorescent Dye using the Green Fluorescent Cell Linker Kit for General Cell Membrane Labelling (Sigma-Aldrich) according to the manufacturer's protocol. Briefly, ExoQuick pellets were resuspended in PBS, 1 ml of Diluent C (CGLDIL, Sigma-Aldrich) was then added to each sample. As a control, 1 ml of Diluent C after adding the same volume of PBS was used. Next, 4 µl of PKH67 dye was added to 1 ml of Diluent C then mixed with the exosomes and the control, PKH67/Diluent C mixture was ultra-centrifuged

before being added to samples. The samples were allowed to incubate < 5 min on a rotor plate. One ml of 1% BSA was then added to bind excess dye. Samples were ultra-centrifuged at 110, 000 xg for 70 min, washed and centrifuged again. For exosome quantification, nanoparticle tracking analysis (NTA) with the ZetaView PMX110 (Particle Metrix) was used. Briefly, exosomes were resuspended in PBS. Two ml of appropriately diluted samples were injected into the ZetaView cell. The instrument was set to obtain NTA measurements at 11 positions, two cycles at each position. During acquisition, temperature was set to 23 ℃, camera sensitivity to 82, 30 frames/s, and shutter speed to 250. Polystyrene beads (100 nm) were used for instrument calibration. For exosome incubation with ceramide analogs N-oleoyl serinol (S18 or bis palmitoyl ethanolamine (B16) the exosomes prepared from 5xFAD or control serum were incubated at 37 °C for 16 h with 50 μM S18 or B16. In addition to the ExoQuick exosome isolation method, we used the Exoeasy Maxi kit (Qiagen, Germany) to isolate exosomes from sera following the manufacturer's protocol. Briefly, sera were diluted with an equal volume of distilled water to reduce viscosity and they were passed through a 0.45 μm filter to remove larger particles. 1 volume of Exoeasy binding buffer (XBP) was then added to 1 volume of sample. Sample/ XBP mix was added onto the Exoeasy spin column and centrifuged at 500 x g for 1 min. Flow-through was discarded and the columns were placed back into the same collection tube. Ten ml Exoeasy washing buffer (XWP) were then added to columns, followed by centrifugation at 500 x g for 5 min to remove residual buffer from the column. Flow-through together with the collection tube were discarded. Spin columns were transferred to fresh collection tubes. Four hundred μl of elution buffer were added to the

membrane and incubated for 1 min, followed by centrifugation at 500 x g for 5 min to collect the eluate.

Brain exosome isolation: This method is a modification to the protocol described by Miltenyi Biotic for isolation and cultivation of astrocytes from adult mouse brain utilizing gentleMACS Octo Dissociator. Briefly, mice were anesthetized using isoflurane inhalation in a chamber followed by perfusion of the whole body with cold 1x PBS to remove blood-derived exosomes from the brain. Mice brains were collected, washed with 1x PBS and cut into eight sagittal slices using sterile scalpel in a petri dish. Brain slices were then transferred to C tubes containing enzymatic dissociation buffer. C tubes were tightly closed and attached upside down onto the sleeves of the gentleMACS Octo Dissociator with Heaters, Program 37C_ABDK_01 being used. Samples were resuspended and applied to a MACS SmartStrainer (70 μm) placed on a 50 mL tube. 10 mL of cold D-PBS were applied onto the MACS SmartStrainer (70 μm). Cell suspensions were centrifuged at 300×g for 10 min at 4 °C, supernatants were carefully transferred to a fresh tube to proceed with exosome isolation. Supernatants were centrifuged at 2000×g for 10 min followed by 10,000×g for 30–40 min then passed through a 0.45 μm filter before following the Exoeasy exosome isolation protocol as described above.

Immunocytochemistry: N2a, primary cultured neurons, or human neuroprogenitor cells were seeded on poly-L-lysine coated cover slips at a density of 25,000 cells/cover slip. N2a cells were allowed to differentiate by gradual serum deprivation[234]. Two days prior to exosome incubation, exosome-free FBS (EXO-FBS - System Biosciences, Mountain View, CA, USA) was used to supplement the media. Cells were then incubated with exosomes and washed three times with PBS, followed by fixation with 4% p-formaldehyde

containing 0.5% glutaraldehyde in PBS for 15 min at room temperature. Permeabilization was performed by incubation with 0.2% Triton X-100 in PBS for 5 min at room temperature. Non-specific binding sites were blocked with 3% ovalbumin/PBS for 1 h at 37 °C. Cells were then incubated with primary antibodies at 4 °C overnight. The next day, cells were washed with PBS and incubated with secondary antibodies diluted 1: 300 in 0.1% ovalbumin/PBS for 2 h at 37 °C. Secondary antibodies were Cy2-conjugated donkey anti-mouse IgM, Alexa Fluor 546-conjugated donkey anti-rabbit IgG, and Alexa Fluor 647-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). After washing, cover slips were mounted using Fluoroshield supplemented with DAPI (Sigma-Aldrich) to visualize the nuclei. We used the following primary antibodies: anti-ceramide rabbit IgG (1:100, our laboratory), anti- flotillin-2 mouse IgG (1:300 BD Biosciences, California, USA, 610383), anti-amyloid-beta mouse IgG 4G8 clone (1:200 Biolegends, California, USA, SIG-39220), beta amyloid recombinant rabbit monoclonal antibody (H31L21, Thermo Fisher), anti-GFAP mouse IgG (1:500, abcam, Cambridge, MA, USA, ab10062), anti-Tom 20 rabbit IgG (1200, Santa Cruz, sc-11415), anti-VDAC1 rabbit IgG (1500, Abcam, ab15895). Fluorescence microscopy was performed using Eclipse Ti2-E inverted microscope system (Nikon, New York, USA). Images were processed using Nikon NIS-Elements software equipped with a 3D deconvolution program. Pearson's correlation coefficient for two fluorescence channels in overlays was used to assess the degree of colocalization.

Proximity ligation assay: Cells were grown and treated as described above in the protocol for immunocytochemistry. Non-specific binding sites were blocked with Duolink PLA blocking solution (Sigma-Aldrich) for 1 h at 37 °C. The primary antibodies used were;

anti-Aβ mouse IgG (1:500 4G8, Biolegends, California, USA, SIG-39220), anti-VDAC1 rabbit IgG (1:1000 abcam, Cambridge, MA, USA, ab34726) Secondary PLA probes: anti-mouse MINUS affinity-purified donkey anti-mouse IgG (H + L) and anti-rabbit PLUS affinity-purified donkey anti-rabbit IgG (H + L) were diluted 1:5 in antibody diluent buffer and samples incubated for 1 h at 37 °C followed by ligation and amplification steps as described in the manufacturer's protocol (Duolink, Sigma-Aldrich). Cover slips were mounted using Fluoroshield supplemented with DAPI (Sigma-Aldrich) to visualize the nuclei. Images obtained with secondary antibody only were used as negative controls representing the background intensity in a laser channel. ImageJ software (https://imagej.nih.gov/ij/) was used to analyze the pictures. Two channels (DAPI and TRITC) were separated to analyze nuclear staining (DAPI) of the images separately from the TRITC channel associated with the PLA dots. Firstly, threshold was set in order to identify nucleus and to allow for binary conversion (black and white). Morphological function was used to separate touching nuclei. Nuclei were counted and added to the region of interest (ROI) where the appropriate minimum and maximum pixel area sizes were set. In the other channel, the number of dots (PLA signals) in each cell as identified by labeling of nuclei was calculated with the "Measure" command from the ROI manager using single point as an output type.

Isolation of mitochondria: N2a cells were seeded on 100 mm dishes at 35–40% of density, followed by incubation with wild type or 5xFAD serum exosomes. Sixteen hours later, cells were harvested and washed twice with ice-cold PBS. Cell pellets were then transferred into a Dounce homogenizer and disrupted with 2 ml of ice-cold mitochondria extrac- tion buffer [10 mM HEPES, 125 mM sucrose, 0.01% BSA, 250 mM mannitol, 10 mM EGTA,

and protease inhibitors (pH 7.2)]. The homogenates were transferred into a centrifuge tube and cell debris pelleted at 700 x g at 4 °C for 10 min to enrich for mitochondria. Following centrifugation under same conditions, supernatants were transferred to a new ice-cold tube, and then mitochondria pelleted at 10,000 x g for 15 min at 4 °C. The mitochondrial pellet was resuspended in 1 ml of lipid binding buffer [20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA (pH 7.5), and 1% digitonin, supplemented with protein inhibitor cocktail (Roche)]. Complete lysis of mitochondrial membranes was achieved by sonication. Removal of insoluble debris was achieved by centrifugation at 10,000 x g for 15 min at 4 °C. The protein concentration in the supernatants from untreated cells and treated cells was determined using Bio-rad RC DC™ Protein Assay.

FLICA and cytotoxicity assay: The FLICA 660 Poly Caspase Assay Kit (ImmunoChemistry Technologies, Minnesota, USA) was used to determine the presence of early caspase activation. This *in vitro* assay employs the fluorescent inhibitor probe 660-VAD-FMK to label active caspase enzymes in living cells. N2a cells (0.25–1·105) were incubated with exosomes (0.5–1x104 exosomes/cell) for 6 h at 37 °C. The cells were washed twice with PBS and resus- pended in RPMI medium with 10% FBS before staining with 30 × FAM-VAD-FMK for 30 min at 37 °C. Cells were washed with 1 x apoptosis wash buffer prior to being fixed with 4% paraformaldehyde supplemented with 0.5% glutaraldehyde. The assay was then followed by PLA as described above.

For LDH cytotoxicity assays, N2a cells were seeded at a density of 5000 cells/well on 96-well plates in complete culture medium and were allowed to grow to adequate confluency. One day before incubation with exosomes, media were replaced with 2% EV-depleted FBS and kept overnight. Cells were treated for 12 h with 1 x 104 exosomes /cell. LDH release

was detected using the CyQUANT™ LDH Cytotoxicity Assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

Western blot and dot blot: For Western blot analysis, samples were mixed with an equal volume of 2X Laemmli sample buffer. Samples were resolved by SDS gel electrophoresis on polyacrylamide gels and transferred to nitrocellulose membrane (Hybond ECL, Amersham Biosciences, UK). Non-specific binding sites were blocked with 5% fat-free dry milk in PBS containing 0.05% Tween-20 followed by overnight incubation with primary antibodies. For exosome characterization we used CD9, CD63, CD81 rabbit antibodies from ExoAb Antibody Kit (System Biosciences, Inc., Mountain View, CA, USA) after dilution to 1:1000. The following primary antibodies were used for immmunolabeling on Western blots: anti-flotillin-2 mouse IgG (1:1000, BD Biosciences, California, USA, 610383), anti-cleaved caspase-3 rabbit IgG (Cell Signaling, Danvers, MA, USA, #9664), anti-VDAC1 goat poly- clonal IgG (1: 200, Santa Cruz Biotechnology, Inc., CA, USA), anti-Drp-1 mouse IgG1 kappa light chain (Santa Cruz, Dallas, TX, USA, sc-271,583). Signals were detected using either pico or femto chemiluminescent (ECL) horseradish peroxidase (HRP) substrate (Thermo Fisher, Massachusetts, USA). Blot images were developed using Azure c600 system (Azure Biosystems, California, USA).

Exosome immune capturing on beads: affinity purification using ceramide beads

Twenty μL of protein A sepharose conjugated magnetic beads were pre-blocked with FcR Blocking Reagent (MACS, Miltenyi Biotec) for 1 h at room temperature. After 3-times washing with lipid biding buffer [20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA (pH 7.5)], either anti-ceramide rabbit IgG or control non-specific rabbit IgG were immobilized on the beads in 1% BSA. Approximately 2 μg were added to each sample and the reaction kept

mixing overnight on a rotary plate. Next day, beads were washed 3-times and diluted exosome samples were added and allowed to incubate with the beads for 2 h at room temperature. Beads were then collected using magnetic columns and washed 3-times with deter- gent free lipid binding buffer. The beads were incubated with an adequate volume of 2x sample Laemmli buffer, heated at 90 °C for 10 min and processed for immunoblot labeling of GFAP. Aliquots of the flow through were used for dot blots determining Aβ content and the residual sample processed for Western blot using 4 x sample Laemmli buffer. Equal volumes of the samples were then applied to each well for Western blot analysis. 4 μL were used for dot blot with the flow through of each sample.

Mass spectrometric analysis of lipids: Exosomes prepared from serum were taken up in water and ceramide species were quantified in the sphingolipidomic (LC-MS/MS) analysis core facility at the Medical University of South Carolina, Charleston, SC. The lipid concentration was normalized to lipid phosphate and exosome number.

Statistical analysis: Clustering analyses were performed with Particle Explorer V2.1.4 (Particle Metrix Inc., Germany) using the following features (1. Particle size 2. Position 3. Area std. 4. Mean intensity std. 5. Trajectory total distance, std. speed, track time, med-speed, and max-speed). For the lipid analysis, results were analyzed with Two-way ANOVA using ceramide species and genetic background as two independent factors. The effect of exosomes from two sources (e.g., wild type and 5xFAD) with unequal sample sizes or unknown variances were analyzed by unpaired t-test with Welch's correction. When multiple comparisons affected by a potential baseline shift in each sample (e.g., mass spectrometric analysis of ceramide species) were analyzed, we used the Bonferroni correction on One-way ANOVA, a statistical test typically applied to mass spectrometric

analyses to exclude false positives. Other tests such as One-way ANOVA with Student-Newman-Keuls (SNK) post hoc test or Tukey correction for comparison of multiple means were applied when used for similar analyses as described in literature. Results showing $p < 0.05$ were reported as statistically significant. All statistical analysis was done on Graph-pad prism software.

2.3 Results

2.3.1 5xFAD mouse and AD patient serum contains exosomes enriched with ceramide and derived from astrocytes (astrosomes)

Several studies showed that exosomes cross the blood- brain-barrier (BBB) carrying toxic and misfolded protein of CNS origin[215, 235]. These studies also showed that purification of exosomes from serum or plasma allows characterization of exosomes from different cell types in the brain, including astrocytes. We used polymer precipitation and membrane affinity chromatography to isolate exosomes from sera of transgenic mouse model of AD and AD patients as these isolation methods were shown to give consistent results when used with plasma or serum[108, 236, 237]. Due to the limitations in availability of AD patient serum, we first focused on characterization of exosomes prepared from serum of the transgenic mouse model of AD (5xFAD) and wild type littermates with identical genetic background (C57Bl/6). 5xFAD mice overexpress presenilins (PS1) with two FAD mutations (M146L and L286V) as well as amyloid precursor protein (APP) with three FAD mutations (V717I, I716V, and K670N/M671L)[221]. Nanoparticle tracker analyses (NTA, Zetaview) and cluster

analyses software (Particle Explorer, Particle Metrix, Mebane, NC) showed that the number of exosomes in wild type and 5xFAD serum from 9 months old mice was similar (8.47x1011 +/− 3.6x1010 exosomes/250 µl serum vs. 9.14x1011 +/− 5.1x1010 exosomes/250 µl serum, N = 6). While the majority of exosomes from wild type serum was composed of a homogenous population of vesicles with medium size of 100 nm (Fig. 2.1a), exosomes from 5xFAD serum contained an additional vesicle population of larger size accounting for 37 +/- 4% of the total population, indicating aggregate formation (Fig. 2.1b). Immunoblot analysis was used to validate the presence of exosomal markers such as tetraspanin proteins (CD63, C9, and CD81 as well as raft and exosome- associated proteins flotillin-1 and flotillin-2, and the astrocyte marker GFAP (Fig. 2.1c).

Lipid analysis using mass spectrometry (LC-MS/MS) showed that 5xFAD exosomes were enriched with ceramide (4.3 pmoles total ceramide/1011 5xFAD serum exosomes vs. 2.2 pmoles/1011 wild type serum exosomes), particularly C16:0, C18:0, C20:0, 22:0, C24:0, and C24:1 ceramide (Fig. 2.1d and e, N = 3). Normalization to lipid phosphate (Fig. 1d) as well as particle count (Fig. 2.1e) showed similar enrichment, confirming that the ceramide composition was representative for the exosome population in serum. We also determined ceramide composition and GFAP association of exosomes in serum from AD patients. Consistent with the results obtained with mouse serum, the number of exosomes in serum from healthy controls and AD patients was similar (2.05x1011 +/− 5.1x109 exosomes/250 µl serum vs. 1.85x1011 +/− 6.2x109 exosomes/250 µl serum. N = 3). There was a population of larger particles which appeared to be similar to that in 5xFAD serum (Fig. 2.11A). Fig. 2.11b shows that the levels of some of the ceramide specie (C16:0, C18:0, C18:1, C20:0, and C20:1 ceramide) were increased in AD patient

exosomes, while others (C22:0 and C24:0 ceramide) were not. This difference in the ceramide profiles between the 5xFAD mouse and AD patient serum exosomes could be due to differences in the activity of ceramide synthases (CerS) in mice vs. patients. The GFAP level associated with serum exosomes from AD patients was comparable to that of healthy controls (Fig. 2.11C). It is possible that characteristics such as exosome enrichment with GFAP are more profound with 5xFAD serum exosomes because of the severe AD pathology phenotype that may not be completely comparable to that of late onset AD patients. Therefore, our results suggest that the main difference between 5xFAD and AD serum exosomes to those from wild type and human controls is a proportion of exosomes enriched with particular ceramides.

2.3.2 Serum astrosomes are associated with Aβ and sensitive to novel ceramide analogs

To further characterize the proportion of ceramide- enriched exosomes, we used anti-ceramide rabbit IgG immobilized on protein A sepharose beads to separate ceramide-enriched exosomes from other exosome populations in serum. Figure 2.2a shows that GFAP labeling was only found with exosomes bound to the beads, while exosomes in the flow through were GFAP negative. Control rabbit IgG did not bind any serum-derived exosomes confirming specificity of the binding reaction for ceramide-enriched astrosomes. Wild type serum also contained astrosomes retained by anti-ceramide antibody, however, at lower concentration as indicated by weaker immunolabeling for GFAP. NTA analysis showed that retention by anti-ceramide beads reduced the number of exosomes by 2.3+/− 0.3% from wild type and 9.2+/− 0.8% (N = 3) from 5xFAD serum indicating that the

proportion of ceramide-enriched astrosomes in 5xFAD serum is ≃ 4-fold higher than that in wild type serum.

Next, we tested if ceramide-enriched exosomes in serum were associated with Aβ by determining the amount of Aβ retained on anti-ceramide beads vs. that in flow through. Immunolabeling using dot blots showed that only the flow through of beads with control IgG contained Aβ42, while amyloid peptide was retained on anti-ceramide beads (Fig. 2.2b). Consistent with immunolabeling for GFAP, the amount of Aβ was 2.2-fold higher in 5xFAD serum than that from wild type mice (not shown). Since ceramide-enriched exosomes were associated with GFAP as well as Aβ we concluded that 5xFAD serum contained a proportion of astrosomes enriched with ceramide and associated with Aβ.

Enrichment of astrosomes with ceramide suggested that this lipid participates in association of Aβ to astrosomes. This hypothesis is consistent with our previous studies showing that anti-ceramide IgG prevented aggregation of exosomes induced by incubation with Aβ. Figure 2.2c shows that incubation with anti- ceramide IgG abolished the proportion of larger sized vesicles in the preparation of 5xFAD exosomes, similar to the effect of anti-ceramide antibody on aggregation of Aβ-associated astrosomes derived from cell culture media. We also found reduction of vesicle size by 17% (N = 4) when adding the novel ceramide analog N-oleoyl serinol (S18) but not N-palmitoyl bisethanolamine (B16, structures are shown in Fig. 2.11 D) to 5xFAD exosomes, suggesting that S18 is a ceramide mimic that disrupts Aβ association and aggregation of astrosomes, probably by interfering with the ceramide- mediated binding of Aβ to astrosomes.

2.3.3 Astrosomes are taken up by neural cells and transport Aβ and ceramide to mitochondria.

To test if serum-derived exosomes are up taken by neural cells, we incubated neuronally differentiated N2a cells, a mouse neuroblastoma cell line, with exosomes labeled with the fluorescent membrane-binding dye PKH67. N2a cells were used as an *in vitro* model since neuroblastoma cells are an established model for neuronal uptake and biological activity of exosomes[238, 239]. Key results were then confirmed using primary cultured neurons. Fig. 2.12A-C shows that both, wild type and 5xFAD serum-derived exosomes labeled with PKH67 are taken up by N2a cells and primary cultured neurons. Cells incubated with wild type serum-derived exosomes were labeled for ceramide, but not or only weakly for GFAP (Fig. 2.3a and c), while cells incubated with 5xFAD serum exosomes were colabeled for ceramide and GFAP (Fig. 2.3b and c). Since there were no or only few cells that showed increased ceramide signals without being colabeled for GFAP, our data demonstrate that N2a cells effectively take up ceramide-enriched astrosomes.

Next, we tested whether astrosomes transported Aβ into N2a cells. Using immunocytochemistry, we detected Aβ signals in N2a cells incubated with 5xFAD exosomes but not with those from wild type serum (Fig. 2.3d and e). The Aβ signal colocalized with labeling for flotillin-2 (arrows in Fig. 2.3e), suggesting that astrosomes delivered Aβ into N2a cells. To further confirm the validity of these results, we used a proximity ligation assay (PLA) for complex formation between ceramide and Aβ in membrane dye PKH67-labeled exosomes taken up by N2a cells[240, 241]. Fig. 2.13A-D shows that PLA signals colocalized with PKH67 labeling and were only observed in cells incubated with 5xFAD exosomes.

We then tested if serum exosomes from AD patients showed similar uptake characteristics as 5xFAD exosomes. N2a cells incubated with AD patient exosomes showed colocalization of ceramide and flotillin-2 (Fig. 2.4b). While endogenous ceramide and flotillin-2 were detectable in exosome-treated cells, we lowered the pertinent fluorescence signals to that of background to specifically monitor ceramide and flotillin-2 contributed by exosomes. Cells incubated with exosomes from healthy controls showed ceramide and flotillin-2 and ceramide labeling, however, at much lower intensity than cells incubate with AD exosomes (Fig. 2.4a). These results indicated that exosomes from human serum, particularly when derived from AD patients, were taken up and transported ceramide into cells.

Several studies showed that mitochondria are affected by Aβ[42, 242, 243]. Using immunocytochemistry for Aβ and Tom-20, we showed that Aβ was labeled in mitochondria of hippocampal tissue from AD patients (arrows in Fig. 2.14A), suggesting that Aβ is transported to mitochondria in AD brain. To investigate exosome-mediated transport of Aβ, we first tested if 5xFAD serum-derived exosomes are transported to mitochondria. Figure 2.5a, b and e show that the exosome marker flotillin-2 colocalized with the mitochondrion marker Tom-20 in 5xFAD exosome-incubated N2a cells. The flotillin fluorescence signal was analyzed after subtracting intrinsic signals, thereby eliminating the possibility that the colocalization resulted from the fluorescence signal of endogenous flotillin with mitochondria. Colabeling and Pearson's coefficient for colocalization were significantly, but only moderately (about 20%) lower when exosomes from wild type serum were used (Fig. 2.5b and e), suggesting that transport of exosomes to mitochondria is not critically dependent on Aβ association.

We then tested if serum-derived exosomes from AD patients and healthy controls matched for sex, age, and body matrix index (BMI) transported Aβ to mitochondria in neurons differentiated from human iPS cells. Figure 2.5d and f shows that Aβ colocalized with Tom-20 in AD patient exosome-treated N2a cells, while there was no Aβ signal detectable when cells were incubated with serum exosomes from healthy controls (Fig. 2.5c and f). AD patient exosomes labeled with Vybrant CM diI also colocalized with Aβ and Tom-20, demonstrating that exosomes effectively transported Aβ to mitochondria (Fig. 2.5g). Mitochondria appeared to be clustered, suggesting that uptake of AD patient-derived exosomes led to mitochondrial damage in neurons.

2.3.4 Astrosomes induce Aβ-VDAC1 complex formation, which activates caspases

Our observation that 5xFAD mouse and AD patient serum exosomes induced clustering of mitochondria in N2a cells and primary cultured neurons prompted us to investigate if Aβ-associated astrosomes are neurotoxic by inducing mitochondrial damage. To test if astrosomes themselves were neurotoxic we analyzed mitochondrial clustering and fragmentation of neuronal processes, and performed TUNEL assays after incubation of primary cultured neurons from mouse brain with astrosomes, Aβ, and Aβ pre-incubated with astrosomes (Fig. 2.6a-f). The number of TUNEL positive cells was increased by 2.6-fold (Fig. 2.6f) when cells were incubated with Aβ- associated astrosomes, concurrent with mitochondrial clustering (arrows in Fig. 2.6e) and 5.9-fold enhanced fragmentation of neuronal processes (Fig. 2.6c) as determined by β-tubulin labeling. This result showed that astrosomes themselves were only marginally toxic, but they significantly enhanced neurotoxicity of Aβ.

Mitochondrial dysfunction is known to be a critical factor in induction of neurotoxicity leading to neurodegeneration in AD[244-246]. One of the previously described targets for Aβ is mitochondrial voltage-dependent anion channel 1 (VDAC1), a mitochondrial gatekeeper for ADP/ATP and calcium localized in the outer mitochondrial membrane[232, 233]. We tested if astrosome-associated Aβ interacted with VDAC1 and induced mitochondrial dysfunction. PLAs using antibodies to VDAC1 and Aβ showed a 6-fold in- crease in the number of signals indicating complex formation between VDAC1 and Aβ when N2a cells were incubated with exosomes from 5xFAD serum as compared to those from wild type serum (Fig. 2.7a-c). PLA signals were clustered (arrows in Fig. 2.7b) consistent with mitochondrial clustering induced by Aβ-associated astrosomes. Mitochondrial damage was confirmed by upregulation of the fission protein Drp-1 in mitochondria isolated from N2a cells incubated with 5xFAD serum exosomes (Fig. 2.7d). Figure 2.7e and f shows that in N2a cells and primary cultured neurons incubated with exosomes from 5xFAD mice or AD patient serum, PLA signals were colocalized with Tom-20, concurrent with mitochondrial clustering. These results confirm that astrosome-associated Aβ formed complexes with mitochondrial VDAC1 and leads to mitochondrial damage. PLA signals for VDAC1-Aβ complexes were also found in the vicinity of amyloid plaques of AD brain tissue, suggesting that VDAC1-Aβ complex formation contributes to AD pathology *in vivo* (Fig. 2.14B).

Since 5xFAD mouse and AD patient serum exosomes transported ceramide into cells (Figs. 2.3b and 2.4b) we tested if VDAC1-Aβ complex formation was colocalized with ceramide. Figure 8a-c shows that in primary cultured neurons incubated with 5xFAD serum exosomes (Fig. 2.8b) or AD patient exosomes (Fig. 2.8c), PLA signals

for formation of complexes of VDAC1 with Aβ were colocalized with ceramide (arrows). Neurons incubated with wild type serum exosomes showed no or only a few PLA signals and they were not colocalized with ceramide (Fig. 2.8a). This result suggested that VDAC1-Aβ complex formation was associated with ceramide derived from exosomes.

Next, we tested if exosome-mediated VDAC1-Aβ complex formation led to activation of caspase 3, a hallmark of neurotoxicity and apoptosis. Figure 2.9a and b shows that in N2a cells incubated with AD patient serum (Fig. 2.9a) or 5xFAD mouse serum (Fig. 2.9b) exosomes, PLA signals for VDAC1-Aβ complexes were colocalized with labeling for activation of caspases (FLICA assays), suggesting induction of apoptosis.

Activation of caspases was confirmed by immunoblot analysis for cleaved caspase 3 (Fig. 2.9d and e). Since the Aβ content of 5xFAD serum exosomes was approximately 25 pg Aβ42/10^{12} exosomes (calculations based on ELISA data, not shown), and 1 x 104 exosomes/cell were added to 1 x 10^5 cells in 1 ml of medium, the apparent Aβ concentration was 5 fmoles/l, which is several orders of magnitude less than what is commonly used in Aβ neurotoxicity assays. VDAC1-Aβ complex formation concurrent with caspase 3 activation was confirmed with 5xFAD serum exosomes and primary cultured neurons (Fig. 2.9c), suggesting that association of Aβ to ceramide-enriched exosomes enhances Aβ neurotoxicity by inducing mitochondrial damage and caspase 3 activation.

Finally, we compared neurotoxicity of 5xFAD serum exosomes with those from mouse brain tissue before and after removal of ceramide-enriched astrosomes using pull down with anti-ceramide antibody. Exosomes were isolated from brain tissue after perfusion with PBS to rule out any contamination with serum exosomes. Analyses using

NTA showed that brain tissue-derived exosomes from wild type and 5xFAD mice were similar in number (1.5×10^9 exosomes/mg wild type vs. 1.6×10^9 exosomes/mg 5xFAD brain tissue) and size distribution (Fig. 2.10a). Normalized to exosome number, the wild type and 5xFAD exosomes contained comparable levels of exosome markers, however, GFAP levels were higher in 5xFAD exosomes indicative of a higher proportion of astrosomes in 5xFAD brain tissue (Fig. 2.10b).

Next, we tested if brain tissue-derived exosomes showed similar effects on neuronal apoptosis as serum exosomes. Results of the FLICA assay (Fig. 2.10c) show that induction of apoptosis by incubation with 5xFAD brain exosomes (0.5×10^4 exosomes/cell) was about 3- fold higher than that with wild type brain exosomes, consistent with data obtained with exosomes from serum (Fig. 2.9). When testing cytotoxicity using a CyQuant assay we found that normalized on the same number of exosomes used per cell ($1x10^4$ exosomes/cell) toxicity of exosomes from 5xFAD brain was 4-fold higher than that from wild type brain and 3.5-fold higher than that from 5xFAD serum exosomes. This result shows that the number of neurotoxic exosomes is highly elevated in 5xFAD brain and that serum exosomes represent a portion of brain exosomes with increased neurotoxicity. When we removed the portion of ceramide-enriched exosomes from 5xFAD and wild type brain tissue and serum, cytotoxicity dropped by half, demonstrating that enhancement of Aβ neurotoxicity is mediated by enrichment of Aβ-associated astrosomes with ceramide.

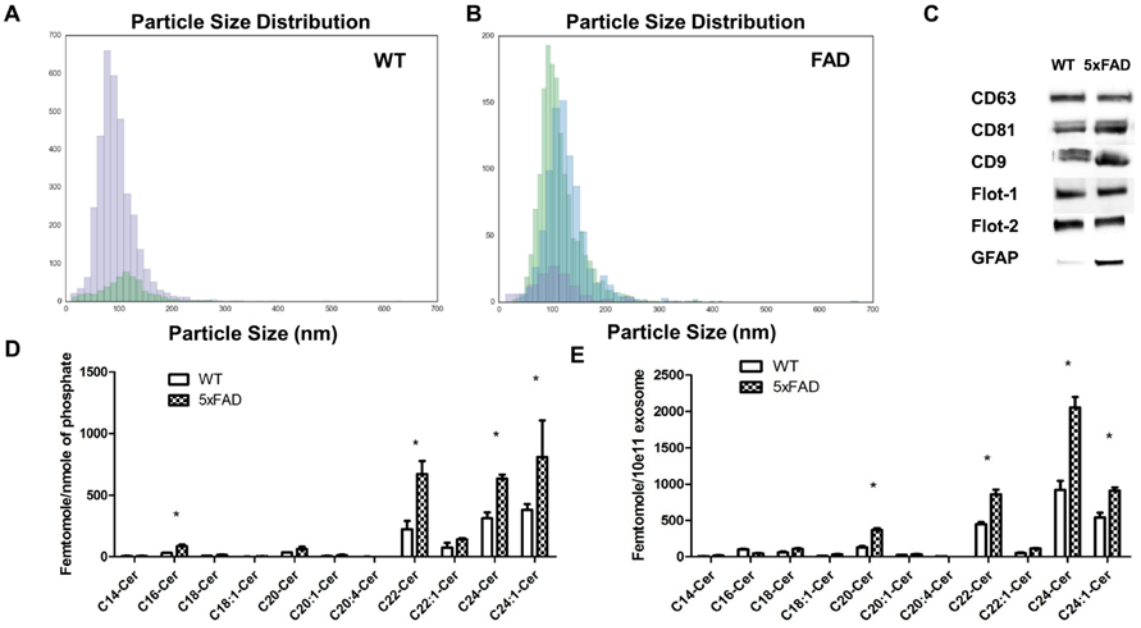

Figure 2.1: 5xFAD serum-derived exosomes are enriched with ceramide and associated with GFAP. (a-b) Cluster analysis of wild type (WT) and 5xFAD serum-derived exosomes after Nano Particle Tracking analysis showing a population of larger exosomes in 5xFAD serum. N = 3 (c) Immunoblot of exosome markers CD9, CD63, CD81, flotillin-1, and flotillin-2, demonstrating higher amounts of GFAP in 5xFAD exosomes compared to WT exosomes. d-e Ceramide species profile determined using LC-MS/MS of WT and 5xFAD serum-derived exosomes and normalized to lipid phosphate content (d) and to exosome count (e) Asterisks denote significance ($p < 0.05$) after Two-way ANOVA followed by Bonferroni correction (N = 3).

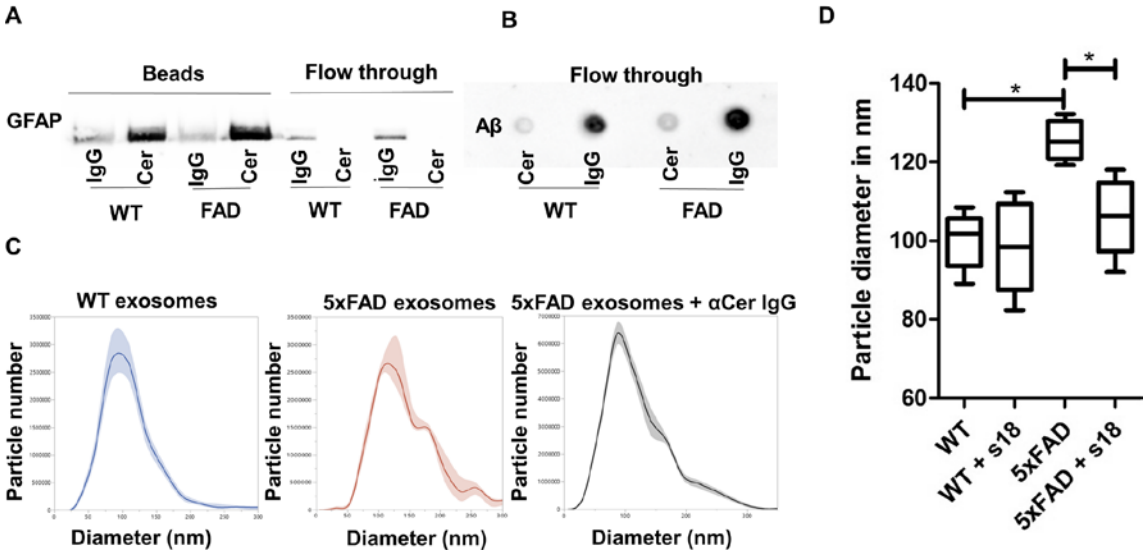

Figure 2.2: 5xFAD serum astrosomes associated with Aβ form aggregates, which is reduced by the novel ceramide analog S18. (A) Gel electrophoresis after immune capturing of exosomes on beads using either ceramide antibody or control IgG and probing with anti-GFAP antibody. Blot is representative of the results from three independent experiments. (B) Dot blot against Aβ using flow through for the same experiment. (C) Size distribution of wild type (WT), 5xFAD, and 5xFAD exosomes treated with anti-ceramide IgG. (D) Size distribution of WT, 5xFAD, and 5xFADexosomes treated with the novel ceramide analog S18. Particle diameter of each sample is represented as ±SEM, two-way ANOVA, *$p < 0.05$. N = 4

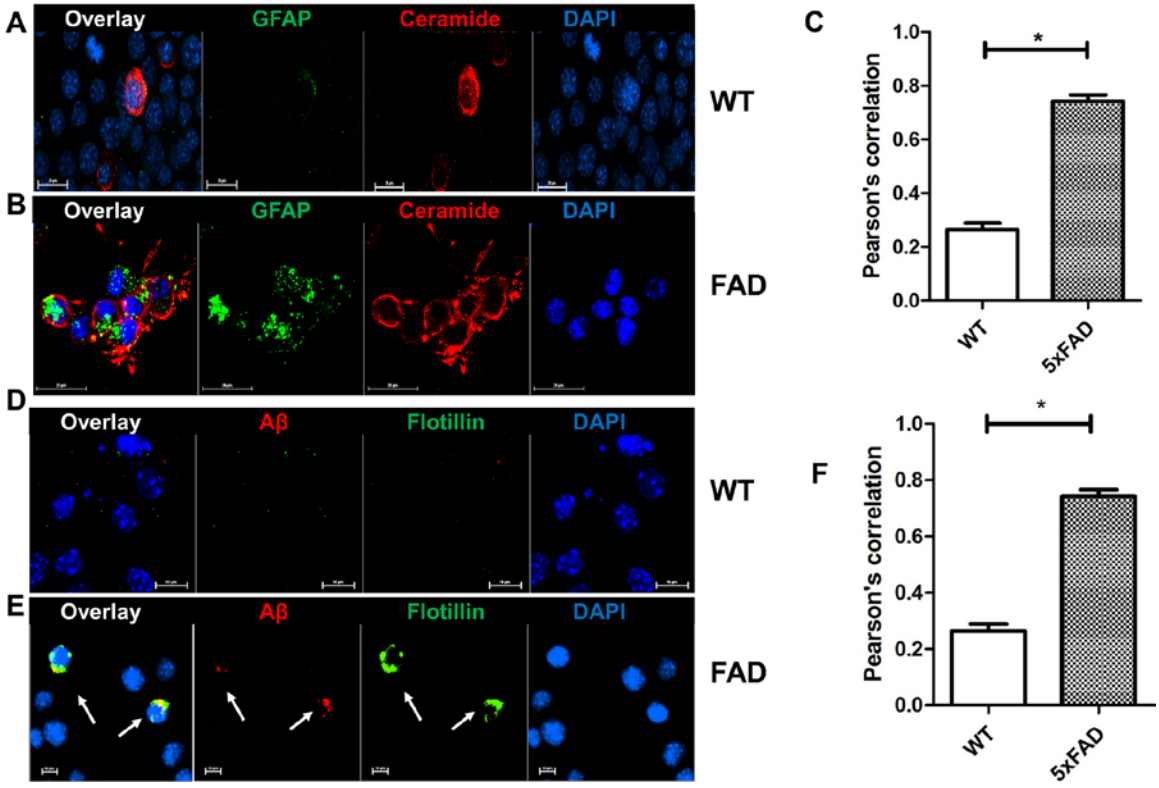

Figure 2.3: 5xFAD serum contains Aβ-associated astrosomes that are taken up by neural cells. Representative images of N2a cells incubated with exosomes isolated from wild type (WT) (a, d) or 5xFAD serum (b, e)and coimmunolabeled with antibodies against GFAP and ceramide (a, b) orflotillin-2 and Aβ (d, e). Arrows point at cells with uptake of Aβ-associated exosomes. The Pearson's correlation coefficient was calculated to compare colocalization of GFAP and ceramide (c) or flotillin-2 and Aβ signals (f) in WT (open bar) and 5xFAD (closed bar). Welch's t-test, *$p < 0.05$. N = 6

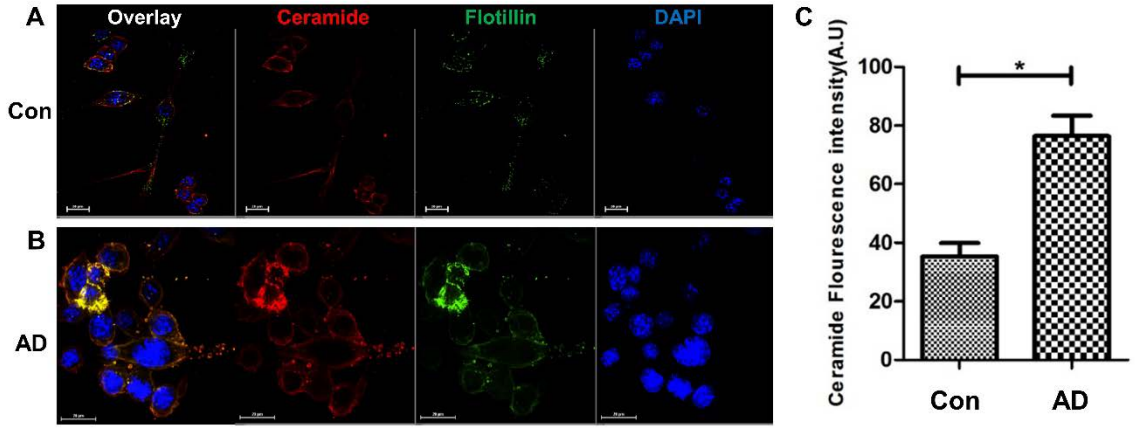

Figure 2.4: Serum-derived exosomes from AD patients transport ceramide into cells. Immunofluorescence images of N2a cells incubated with either (a)healthy control or (b) AD patient serum-derived exosomes labeled with anti-ceramide and flotillin-2 antibodies. (c) fluorescence intensities for the ceramide signal. N = 6. Student t-test with Welch's correction, *$p < 0.05$, **$p < 0.01$.

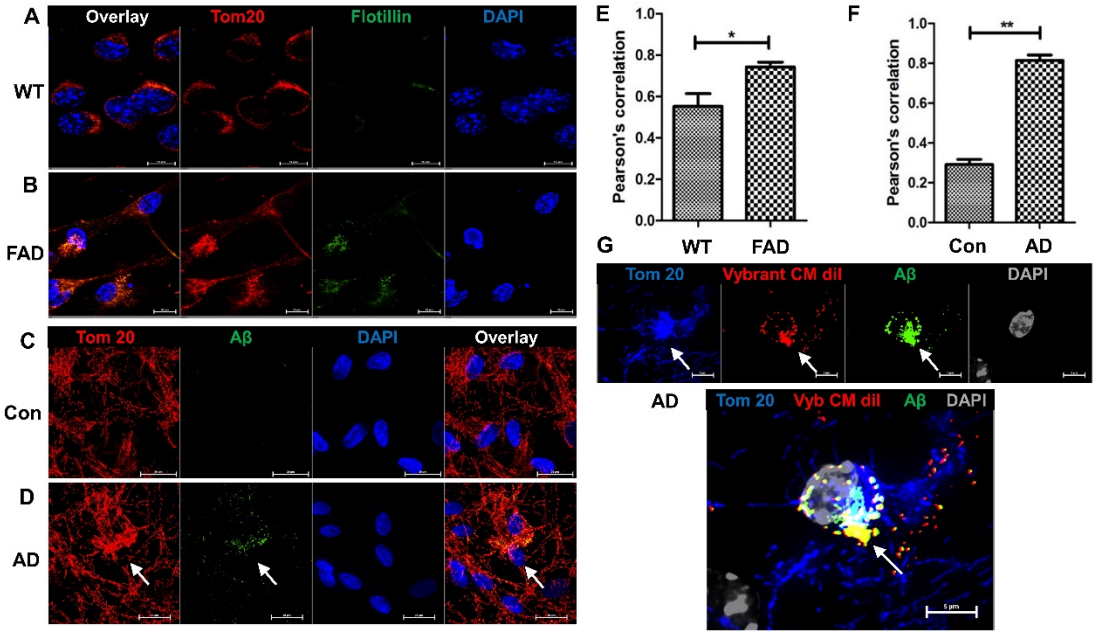

Figure 2.5: Serum-derived exosomes from 5xFAD mice and AD patients' shuttle Aβ to mitochondria in N2a cells and neurons. Immunofluorescence images of N2a cells incubated with either (a) wild type or (b) 5xFAD serum-derived exosomes and then labeled with flotillin-2 and Tom-20 antibodies. (e)Pearson's correlation coefficient for colocalization of flotillin-2 and Tom-20. N = 6. Student t-test with Welch's correction. *p < 0.05. c, d Neurons differentiated from human iPS cells and incubated with control healthy human (c) or AD patient exosomes (d) showing that only AD patient exosomes shuttle Aβ to mitochondria in neurons (arrows). f Pearson's correlation coefficient for colocalization of Aβ with Tom-20. N = 6. Student t-test with Welch's correction. **p < 0.01. g as in d, but additional labeling of AD patient exosomes with Vybrant CM diI showing mitochondrial clustering (arrows) induced by Aβ-associated exosomes. Bottom image shows detail of (g)

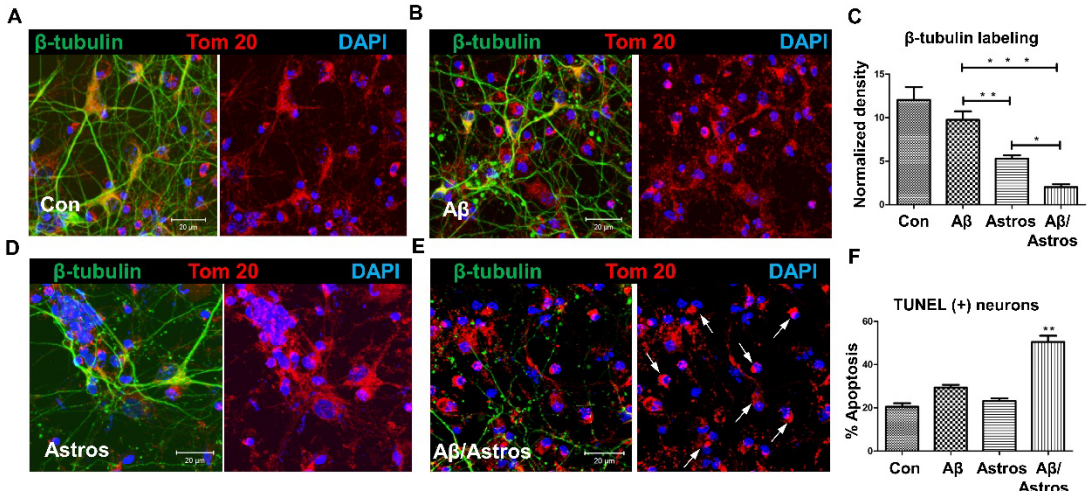

Figure 2.6: Neurotoxic effect of Aβ42/astrosome complexes on primary neuronal cultures. Representative single-focal-plane images of β-tubulin and Tom-20 labeling obtained with control (a), Aβ42 (b), astrosome (d), or Aβ42/astrosome-incubated (e) primary cultured mouse neuron. Arrows indicate mitochondrial clusters. c Average normalized density of β-tubulin labeling reveals that the greatest loss occurs in cultures treated with Aβ42/astrosome complexes. N = 6. One-way ANOVA with Student-Newman-Keuls (SNK) post hoc test). ***$p < 0.001$, **$p < 0.005$, *$p < 0.05$). f TUNEL assay detected a 2.6-fold increase in neuronal cell death when Aβ42 and astrosomes were combined (N = 4. One-way ANOVA with SNK post hoc test)

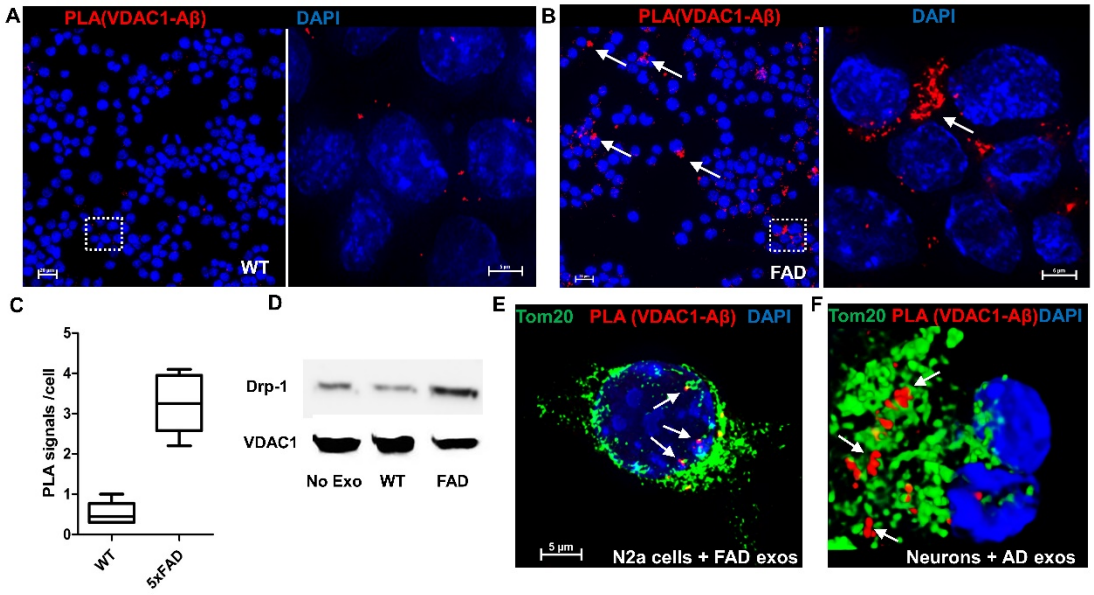

Figure 2.7: Aβ-associated exosomes mediate complex formation between VDAC1 and Aβ and induce mitochondrial damage. a, b Representative immunofluorescence images of N2a cells incubated with wild type exosomes (a) or 5xFAD exosomes (b) showing increased number of PLA signals in cells incubated with 5xFAD exosomes. Right panels show detail from left panel (frame). Each red dot denotes complex formation between Aβ and mitochondrial VDAC1. Arrows indicate PLA signals in mitochondrial clusters (c) Calculation and comparison of average PLA signals per cell between wild type and 5xFAD incubations, six images for each condition. Student t-test followed by Welch's correction. N = 6. p <0.001 (d) Western blot of mitochondrial protein from N2a cells using antibody against Drp-1 and VDAC1 as a reference protein. e, f Colocalization between PLA signals for VDAC1-Aβ complexes and mitochondrial marker Tom-20 labeling in N2a cells and primary cultured neurons incubated with 5xFAD serum exosomes (e) and AD patient serum exosomes (f).

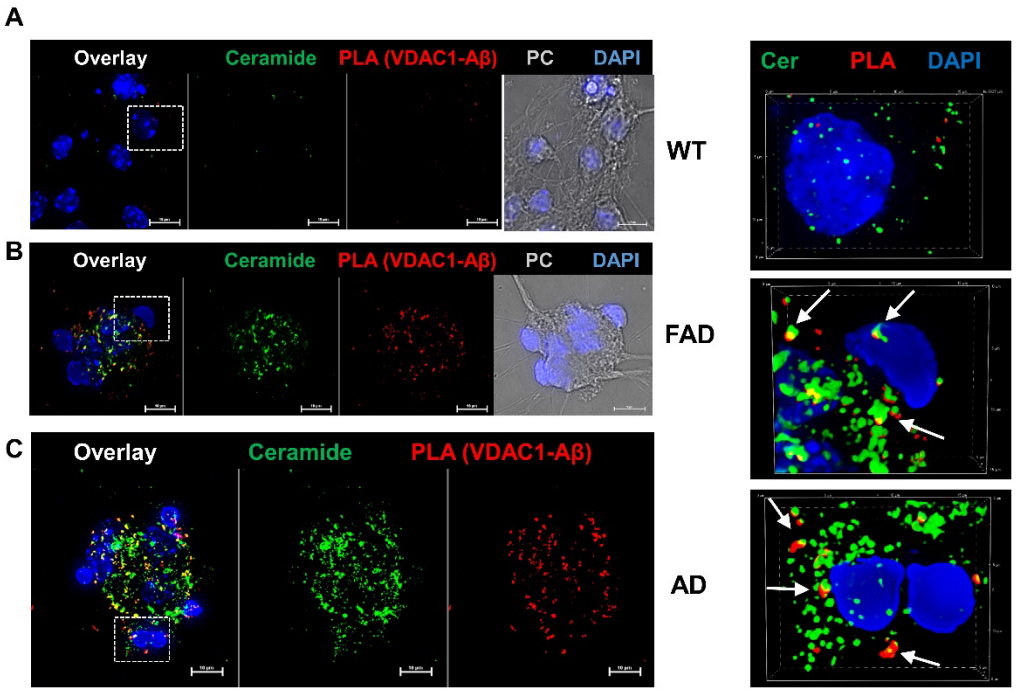

Figure 2.8: Exosome-induced VDAC1-Aβ complex formation is associated with ceramide. Representative immunofluorescence images for PLA signals fromVDAC1-Aβ complexes and ceramide in primary cultured neurons incubated with wild type mouse exosomes (a), 5xFAD mouse exosomes (b), or human AD patient serum-derived exosomes (c). Images in right panel are details at higher magnification (frames in left panel) with arrows pointing at PLA signals colocalized with ceramide

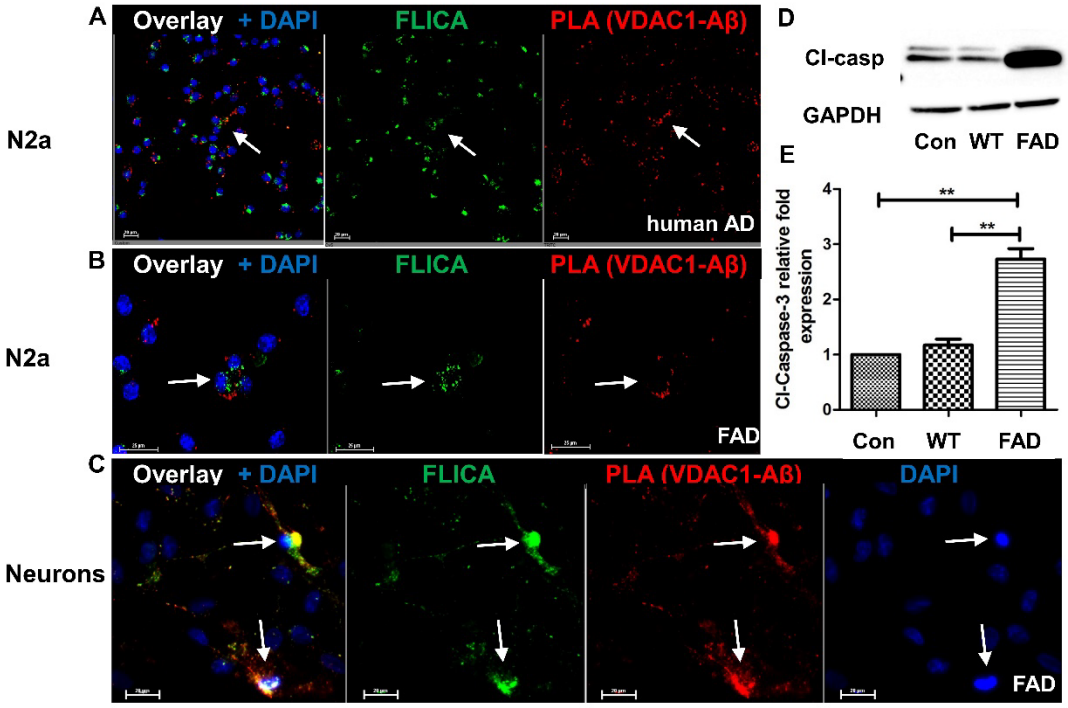

Figure 2.9: 5xFAD and human AD patient serum-derived exosomes trigger apoptosis in cells induced by interaction between mitochondrial VDAC1 and Aβ. Representative immunofluorescence images of N2a cells incubated with (a) AD patient exosomes or (b) 5xFAD mouse serum-derived exosomes. FLICA assays were followed by PLAs for VDAC1-Aβ complex formation. Images show that cells with VDAC1-Aβ complexes undergo apoptosis(arrows). c Primary cultured neurons incubated with 5xFAD serum exosomes followed by FLICA assays and PLAs. Arrows indicate neurons colabeled for VDAC1-Aβ complexes and caspase 3 activation. These cells show pyknic nuclei (condensed DAPI labeling) indicative of apoptosis. (d)Western blot with N2a cell lysate immunolabeled for cleaved caspase 3 using GAPDH as a reference protein. Blot is representative of three independent experiments. e Relative fold expression of cleaved caspase 3 normalized to GAPDH. One-way ANOVA followed by Tukey correction. N = 3. **$p < 0.001$

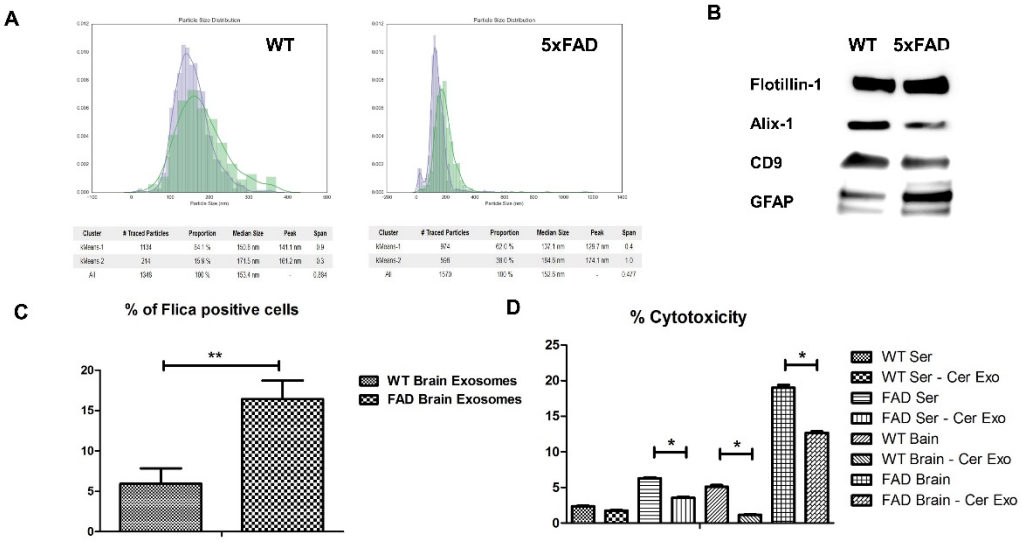

Figure 2.10: Ceramide-enriched 5xFAD brain tissue and serum exosomes are neurotoxic. (a) Cluster analysis of wild type (WT) and 5xFAD brain tissue derived exosomes after Nano Particle Tracking analysis. N = 3 (b) Immunoblot of exosome markers flotillin-1, Alix-1, CD9, and GFAP, demonstrating higher amounts of GFAP in 5xFAD exosomes compared to WT exosomes. c FLICA assay shows 3-fold increased rate of apoptosis induction (cells with activated caspase 3) after incubation for 12 h of N2a cells with 5xFAD brain tissue exosomes compared to WT exosomes .N = 15, Student t-test with Welch's correction $p < 0.005$. d Cytotoxicity (CyQuant) assay shows reduction of neurotoxicity after depletion of ceramide-enriched exosomes. N = 7, Multiple Student t-test with Welch's correction $p < 0.05$.

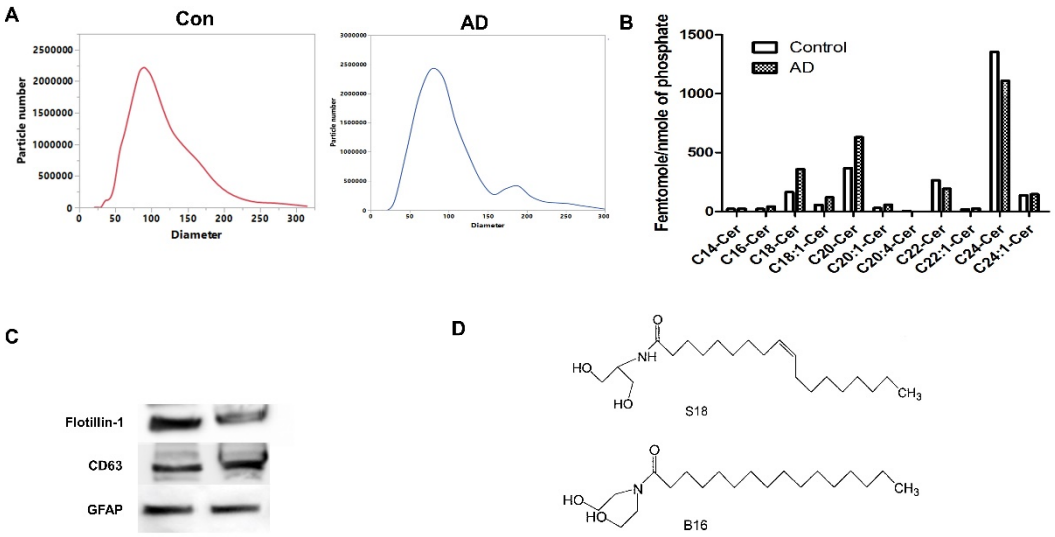

Figure 2.11: Serum-derived exosomes from AD patients are enriched with ceramide. (A) Size distribution (Zetaview NTA analysis) of human serum exosomes. (B) Ceramide species profile using lipid mass spectrometry (LC-MS/MS) of AD patient serum-derived exosomes normalized to phosphate content. (C) Immunoblot for exosome markers CD63 and Flotillin-1 showing equal protein expression levels of GFAP in AD and healthy control individuals. (D) Structures of ceramide analogs S18 and B16.

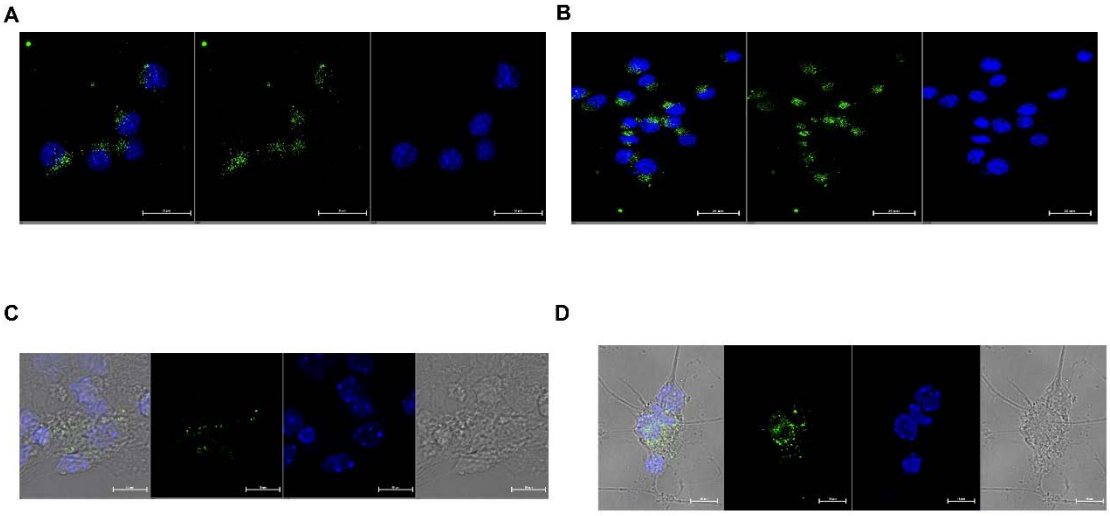

Figure 2.12: Serum derived exosomes from WT and 5xFAD mice are taken up by N2a cells. Representative fluorescence microscopy images of PKH67-labeled exosomes from wild type (A) and 5xFAD (B) mice showing their uptake by N2a cells and primary cultured neurons (C, wild type; D, 5xFAD exosomes).

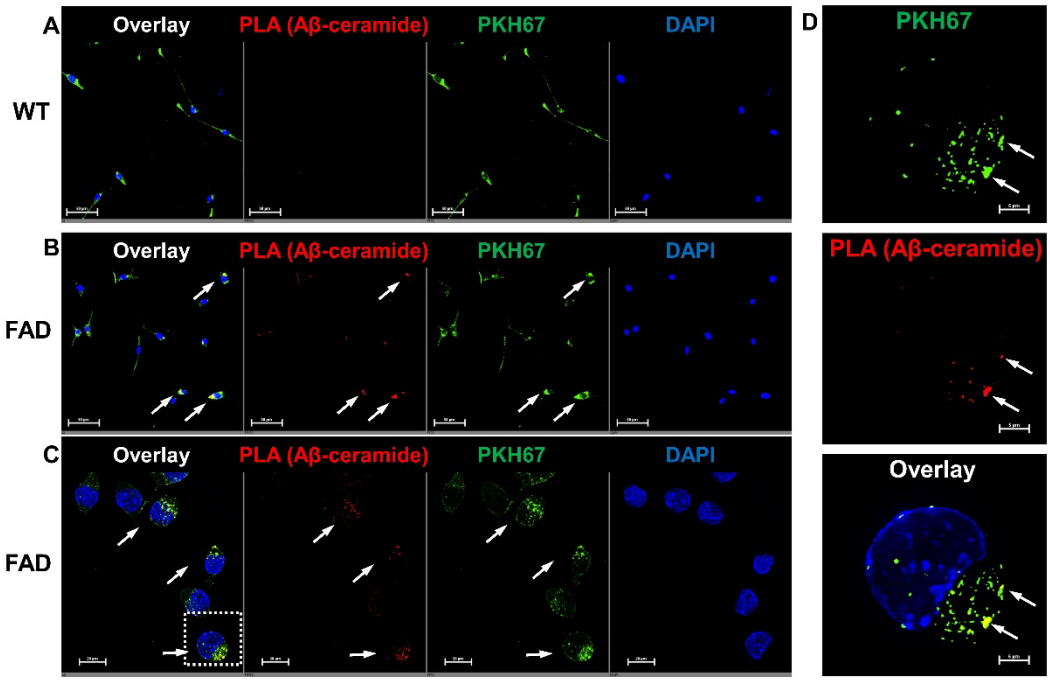

Figure 2.13: 5xFAD exosomes retained complex formation between Aβ and ceramide after uptake into N2a cells. Either wild type (A) or 5xFAD (B, C, D) serum-derived exosomes were labeled with PKH67 dye and then then used for incubation of N2a cells. PLA shows complex formation between Aβ and ceramide only with 5xFAD exosomes. C is similar to B at higher magnification. D is detail (frame) from C.

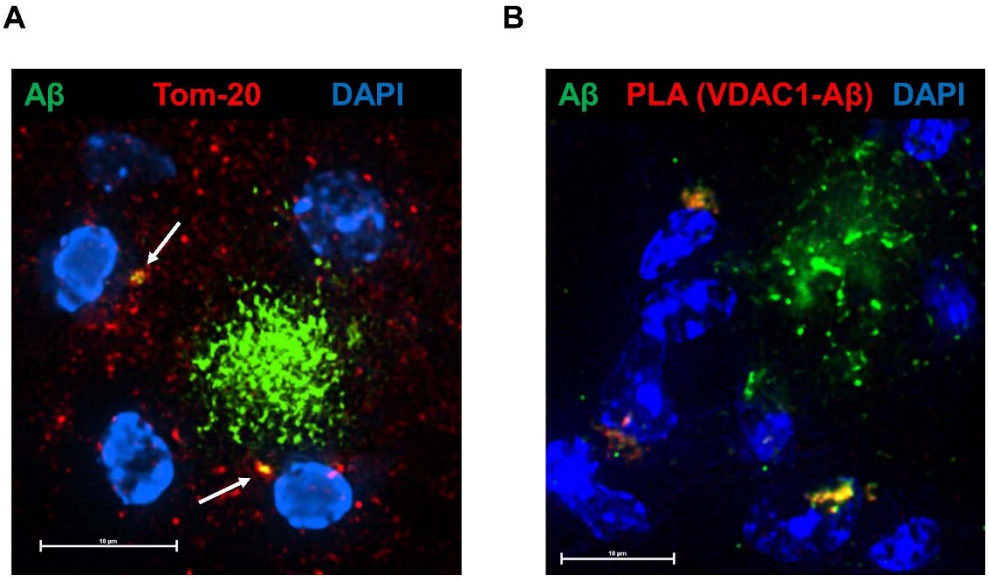

Figure 2.14: Interaction between Aβ and mitochondrial via VDAC1 in human brain. (A) Representative flouresence image of human brain section showing colocalization of Aβ with mitochondrial Tom20 aroung amyloid plaque (arrows). (B) PLA using antibodies against Aβ and mitochondrial VDAC1 showing complex formation in cells surrounding amyloid plaque.

2.4 Discussion

While accumulation of Aβ is a hallmark in human AD, its causal role in neurotoxicity and cognitive decline is persistently elusive. Other factors such as tau, bacterial or viral infection, insulin resistance, and neuroinflammation are invoked in AD, and yet none of these factors was proven critical in the onset of the disease or neurodegeneration[247-249]. Probably the most likely explanation for AD pathophysiology is a multifactorial cascade of events with any of these factors initiating or amplifying each other during the course of the disease. This multifactor hypothesis implies that each factor is necessary, but not sufficient to initiate AD or cause neurotoxicity. The idea that Aβ or tau require additional factors critical to mediate or enhance their neurotoxicity is not surprising. Many studies showed that Aβ and tau concentrations used to induce neuronal damage or death *in vitro* are often orders of magnitude higher than those found *in vivo*[16, 250, 251]. In addition, Aβ and tau concentrations or plaque and tangle size *in vivo* are often not correlated with the extent of neurodegeneration or cognitive decline[16]. Recently, extracellular vehicles (EVs), exosomes or microvesicles, were proposed as carrier for transport and uptake of Aβ and tau into neurons[190, 202]. However, it is not clear how this uptake may lead to neurodegeneration in AD. In this study, we show for the first time that exosomes are not only carrier for Aβ, but also sensitize neurons to Aβ toxicity. Several studies showed that plasma or serum from AD mice and patients contains exosomes that are associated with Aβ, demonstrating that Aβ-associated exosomes crossed the blood brain barrier (BBB)[217, 252]. In AD patients, about 23% of these exosomes were found to be derived from astrocytes, while the remainder was from neurons. Association of astrocyte-derived

exosomes (termed astrosomes in the current study) with Aβ42 was shown to be several-fold higher than that of neurons, suggesting that the primary source of Aβ-associated exosomes are astrocytes[253]. It was not investigated, however, if Aβ-associated astrosomes were enriched with ceramide or taken up by cells and involved in the pathophysiology of AD. Using a method developed in our laboratory, lipid-mediated affinity chromatography (LIMAC) of vesicles with anti-ceramide antibody[254], we showed for the first time that astrosomes from serum were ceramide-enriched and associated with Aβ. Nano- particle tracking analysis (Zetaview) of LIMAC fractions showed that 9.2% of serum-derived exosomes were Aβ- associated astrosomes, while the remainder (not bound by anti-ceramide antibody) were only weakly labeled for GFAP and likely of neuronal origin. The Aβ content in these vesicles was approximately 25 pg Aβ42/10^{12} exosomes, which corresponded to 250 µl of serum (5xFAD mice). Mass spectrometric (LC-MS/MS) analysis of serum-derived exosomes showed enrichment with ceramide species similar to those found in exosomes released by primary cultures of astrocytes *in vitro*[164]. This data prompted us to hypothesize that serum- derived astrosomes associated with Aβ by a mechanism similar to that previously published for *in vitro* generated astrosomes.

In previous studies, we showed that anti-ceramide antibody prevented association of Aβ42 with *in vitro* generated astrosomes [226]. We concluded that ceramide was critical for binding of Aβ42 to astrosomes, by either directly interacting with it or facilitating interaction of Aβ with other components of the vesicle membrane. In this study, we tested a novel concept using ceramide analogs originally developed in our laboratory to disrupt binding of Aβ to astrosomes[255]. The novel ceramide analog N-oleoyl serinol (S18)

reduced the average diameter of exosomes from 5xFAD mice. This result suggests that S18-treated exosomes are less prone to aggregation, and probably, association of Aβ with exosomes is resolved.

To date, only a few studies addressed a potential function of Aβ-associated exosomes in AD. It was shown that exosomes can spread amyloid between neurons and that uptake of EVs isolated from the cerebrospinal fluid or plasma of AD patients impairs mitochondrial respiratory function and induces caspase activation [190, 202]. However, it was not shown that Aβ-associated astrosomes are ceramide-enriched, transported to mitochondria, and mediate Aβ-binding to a critical mitochondrial protein. Our previous studies suggested that Aβ-associated astrosomes induce nucleation of amyloid plaques and critically participate in neurodegeneration [256]. However, consistent with other reports showing that neurotoxicity is not directly correlated with plaque size, we hypothesized that Aβ-associated astrosomes mediate neurotoxicity by a mechanism distinct from plaque formation. The results using exosomes from serum of 5xFAD mice and AD patients show that Aβ-associated astrosomes are transported to mitochondria. This is demonstrated by colabeling of Aβ and ceramide with Tom-20 in cells that are also positive for GFAP and flotillin 2 when exposed to serum exosomes from 5xFAD mice or AD patients, but not from wild type mice or healthy controls. These cells show mitochondrial damage as documented by clustering of mitochondria and increased levels of the mitochondrial fission protein Drp-1. Our data is consistent with that from previous studies reporting that the level of Drp-1 is elevated in AD brain and neurons exposed to Aβ *in vitro* [257, 258]. In our previous studies, we showed that Aβ exposure leads to mitochondrial malformation and dysregulation of VDAC1, the main ADP/ATP transporter in the outer mitochondrial

membrane the level of which is elevated in AD[92, 101, 241, 259]. Our data is consistent with that from previous studies reporting that Aβ binds to VDAC1 and induces formation of a pro-apoptotic pore [260]. Using cortical protein lysates from AD patient and AD mouse model brains, it was shown by co-immunoprecipitation assay that Aβ binds to mitochondrial VDAC1[99]. However, none of the previous studies investigated the effect of Aβ-associated exosomes on VDAC1 and its interaction with Aβ.

To test the role of Aβ-associated exosomes in the interaction of VDAC1 with Aβ we performed proximity ligation assays (PLAs) after exposure of N2a cells and neurons to exosomes from 5xFAD mice and AD patients as well as wild type mice and healthy controls. Our results show that exosomes from 5xFAD mouse or AD patient serum lead to PLA signals indicating formation of a complex between VDAC1 and Aβ. Therefore, we concluded that Aβ-associated exosomes induced or mediated complex formation be- tween VDAC1 and Aβ. Currently, we are investigating the mechanism by which exosomes induce this complex formation. Figure 2.11 shows a model for endocytotic uptake and interaction with VDAC1 at mitochondria mediated by Aβ-associated astrosomes. Aβ-associated astrosomes may either be endocytosed as vesicles or first fuse with the plasma membrane. In both cases, Aβ (red in Fig. 2.11) remains associated with ceramide (green in Fig. 2.11), probably in the form of ceramide-rich platforms, a type of lipid rafts enriched with ceramide[144]. The persistent association with ceramide explains why Aβ and ceramide remain colabeled after uptake of Aβ-associated exosomes into N2a cells and neurons. Next, Aβ is shuttled to mitochondria, which is probably mediated by vesicular transport, either by Aβ-associated endosomes or other types of vesicular compartments such as aberrant autophagosomes[228, 261-263]. Finally, Aβ is imported into

mitochondria to interact with VDAC1, which induces a pro-apoptotic pore that leads to release of cytochrome c and activation of caspases [260]. While interaction of Aβ with VDAC1 and formation of the pro-apoptotic pore was reported, the role of ceramide and exosomes in this process has not yet been investigated.

Ceramide was invoked in neuronal Aβ release and formation or secretion of exosomes from astrocytes[226, 229, 256, 264]. Our studies showed that ceramide is instrumental for interaction of Aβ with astrosomes. In the novel mechanism depicted in Fig. 2.11, ceramide may critically participate in several steps of uptake, transport, and mitotoxicity of Aβ. Firstly, association of Aβ with ceramide in the astrosomal membrane may induce a specific Aβ isoform or aggregate promoting endocytosis. This hypothesis is consistent with our observation that a proportion of exosomes from serum of 5xFAD mice or AD patients form aggregates. Secondly, ceramide may critically participate in neuronal endocytosis and transport of Aβ to mitochondria. In numerous studies, it was shown that fluorescently labeled ceramide was taken up by endocytosis and then transported to specific compartments, mainly the Golgi apparatus[265-267]. Albeit the reason for Golgi accumulation is unclear, other studies support the idea that ceramide guides transport of endosomes to specific compartments, which may include those interacting with mitochondria[268, 269]. It should be noted that our previous studies showed that uptake of Aβ42 by glial cells is reduced by at least 50% when associated with exosomes, suggesting that uptake of Aβ-associated exosomes as observed in our current study is specific for neurons and potentially mediated by ceramide[256]. Thirdly, ceramide may participate in import of Aβ into mitochondria, e.g., by fusing Aβ-associated astrosomes to the outer mitochondrial membrane, and binding to VDAC1. Interaction of either ceramide

or Aβ with VDAC1 was demonstrated by several studies from our and other laboratories[91, 99, 100, 241]. Alternatively, membranes closely associated with the outer mitochondrial membrane such as mitochondria-associated membranes (MAMs) may take part in the interaction of Aβ with VDAC1. Fourthly, ceramide may facilitate formation of a pro-apoptotic pore that is associated with the VDAC1-Aβ complex. VDAC1 oligomers as well as ceramide channels were reported to partake in pro-apoptotic pores at mitochondria[97, 270], however, the involvement of Aβ- associated and ceramide-enriched exosomes in formation of these pores was not discussed yet. Our observation that VDAC1-Aβ complexes are colocalized with ceramide at mitochondria suggests a novel mechanism by which association of VDAC1 with ceramide and Aβ induces or facilitates pro-apoptotic pore formation. Finally, since association o Aβ with exosomes is remarkably stable and persists during passage through the BBB into the blood stream, the proportion of Aβ-associated exosomes may participate in systemic distribution and potentially, reuptake of Aβ and its spreading throughout the brain.

Our data show that 5xFAD brain-derived and ceramide-enriched exosomes are neurotoxic and serum contains a proportion of these exosomes crossing the BBB. However, it is conceivable that these exosomes acquire additional toxic factors during their passage through the blood stream. Therefore, Aβ-associated exosomes in serum may not only be a biomarker and "window" to the brain, but actively participate in spreading AD pathology and contributing to Aβ neurotoxicity after reuptake into the brain. While we utilized serum-derived exosomes in our *in vitro* experiments to elucidate the proposed mechanism, studies are planned to further test the significance of ceramide-enriched exosomes for Aβ neurotoxicity *in vivo*. These studies will address the function of different

ceramide species in neurotoxicity, particularly when comparing 5xFAD mice with AD patients and the *in vivo* significance of systemic distribution and reuptake of Aβ-associated astrosomes. Here we present for the first time experimental evidence for our hypothesis that ceramide and Aβ act synergistically to target VDAC1 and induce caspase activation, ultimately leading to neuronal malfunction and apoptosis. Therefore, Aβ-associated astrosomes assisting in Aβ uptake, transport, and mitotoxicity are a novel key factor in sensitizing neurons to Aβ and a potential pharmacological target to prevent neurodegeneration in AD.

Current pharmacological approaches exclusively aim at interfering with ceramide generation using inhibitors for enzymes in ceramide metabolism [226, 270-272]. The most prominent example is GW4869, an inhibitor for neutral sphingomyelinase 2 (nSMase2) we have shown to reduce plaque formation and improve cognition in male 5xFAD mice [226]. We previously reported that the nSMase2- deficient 5XFAD mice (fro;5XFAD) showed a reduced number of brain exosomes, ceramide levels, glial activation, total Aβ42 and plaque burden, and improved recognition in a fear-conditioned learning task]. In future studies, we will specifically address the function of astrocyte-derived exosomes in Aβ neurotoxicity by including mice with astrocyte-specific deletion of nSMase2 as well as knockouts of individual ceramide synthases.

While enzyme inhibitors are promising as lead compounds interfering with ceramide metabolism in AD, alternative pharmacological approaches targeting ceramide but not depending on enzyme inhibition may offer additional benefits. About 20 years ago, our laboratory designed and synthesized novel ceramide analogs of the β-hydroxy alkylamine type, particularly N-oleoyl serinol [273] (S18) that do not inhibit ceramide

generation, but interfere with binding of ceramide to its protein interaction partners such as atypical protein kinase C λ/ξ [273]. These analogs were shown to be non-toxic to normal cells, but induce apoptosis in cancer cells[255]. We hypothesized that novel ceramide analogs may also interfere with binding of Aβ to ceramide in astrosomes, thereby providing a novel therapeutic approach preventing astrosome-mediated spreading and uptake of Aβ, and sensitization of neurons to Aβ. Our data with S18 obliterating exosome aggregates in 5xFAD serum support this hypothesis, which will also be investigated in our future research. In summary, our data show for the first time that astrosomes sensitize neurons to Aβ and suggest that interfering with binding of Aβ to astrosomes using novel ceramide analogs may provide a novel therapeutic strategy for treating AD.

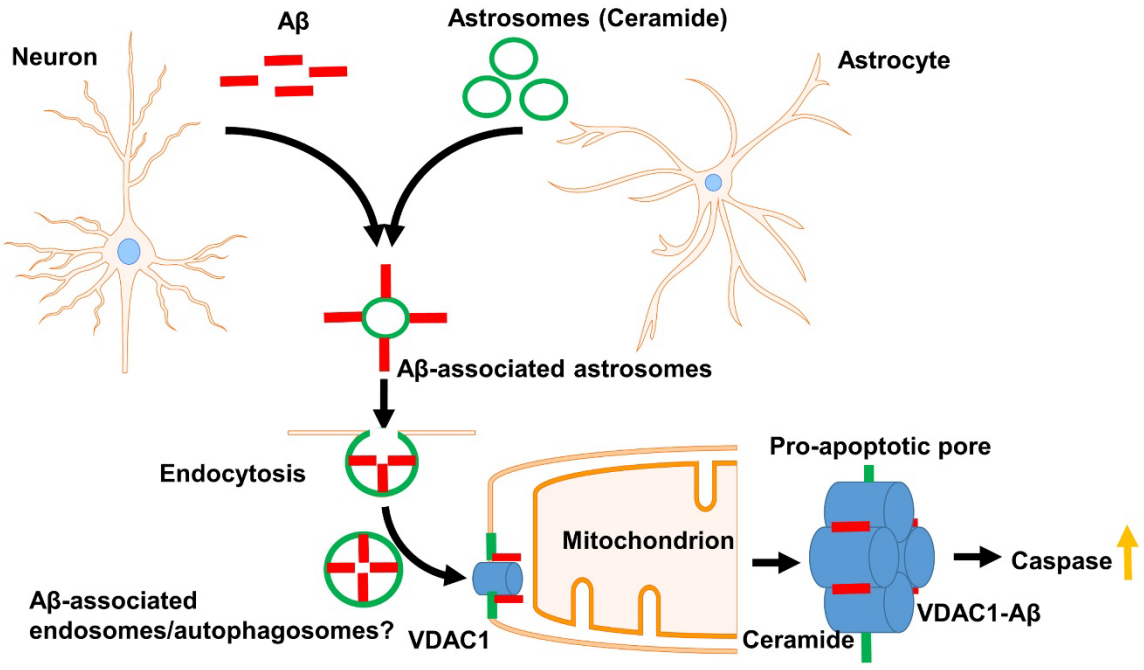

Figure 1.15: Potential mechanism of neurotoxicity induced by Aβ-associated astrosomes. Aβ secreted by neurons (red) binds to ceramide-enriched exosomes secreted by astrocytes (astrosomes, green). Aβ-associated astrosomes are endocytosed by neurons and transported to mitochondria. The vesicles fuse with the outer mitochondrial membrane and mediate binding of Aβ to VDAC1. A pro-apoptotic pore, probably associated with ceramide, is formed which leads to activation of caspases and induction of neuronal cell death

CHAPTER 3. *IN VIVO* EVIDENCE OF EXOSOME-MEDIATED AB NEUROTOXICITY

3.1 Introduction

Alzheimer's disease (AD) is the most prevalent form of dementia in elderly patients[274]. Amyloid beta (Aβ) is one of the most studied contributors to AD pathology, however, the intracellular toxicity mechanism of Aβ is not clear yet. Recently, a wealth of publications has demonstrated ways by which exosomes could participate in the pathology of AD either in a protective manner or as a facilitator for plaque deposition and shuttling of misfolded protein[184, 190, 203, 275, 276]. In our recent work, we showed that serum from the transgenic mouse model of familial AD (5xFAD) and AD patients as well as 5xFAD brain-derived exosomes contains exosomes that are associated with Aβ. The association with exosomes was shown to substantially enhance Aβ neurotoxicity in AD. When taken up by neurons *in vitro*, these Aβ-associated exosomes were transported to mitochondria, induced mitochondrial clustering, and upregulated the fission protein Drp-1. Aβ-associated exosomes mediated binding of Aβ to voltage-dependent anion channel 1(VDAC1) and subsequently, caspase activation. Aβ-associated exosomes also induced neurite fragmentation and neuronal cell death. However, despite solid and convincing results, our study lacked the *in vivo* component. Here, we aim to augment the significance of our previous experiments by adding *in vivo* data that confirms our previous results.

Exosome secretion from multiple CNS cell types has been well established *in vitro*, besides, the detection of exosomal markers from human cerebrospinal fluid (CSF) samples indicates that active secretion of these exosomes in the CNS. However, exosome signaling in situ in the CNS remains essentially unexplored. Aiming to investigate that in the context of AD, we first injected brain-derived exosomes from nine months old 5xFAD

mice into the hippocampus of 3 weeks old mice. Second, we generated an astrocyte-specific exosome reporter mouse that secrete GFP labeled exosomes from astrocytes. This tool allows us to visualize and track the exosomes secreted from astrocytes aiming to understand their dynamics and elucidate their role in AD pathology.

3.2 Methods

Animals: The CD63-GFPf/f knock-in mice were obtained from Dr. Yongjie Yang in Tufts University School of Medicine[277]. ALDH1l1-CreERT transgenic, (#031008), WT mice (C57 BL6 background), and 5xFAD (#34848) were obtained from The Jackson Laboratory. CD63-GFPf/f and ALDH1l1-CreERT mice were bred to generate ALDH1l1-CreERTCD63-GFPf/+ mice. ALDH1l1-CreERTCD63-GFPf/+ mice were then bred with CD63-GFPf/f to generate ALDH1l1-CreERTCD63-GFPf/f mice which was bred with 5xFAD mice. Breeding continued until 5xFAD-ALDH1l1-CreERT-CD63-GFPf/f mice were generated. Mice were kept on a 12 h light/dark cycle with food and water ad libitum. Animal protocols used in this study has been approved by the University of Kentucky IACUC committee.

Drug administration: 4-hydroxytamoxifen (4-OHT) (Sigma) was prepared at 5 mg/mL in Corn oil at was given to animal at a concentration of 20mg/kg. 4-OHT was given intraperitoneal for 5 consecutive days. Mice were kept on heating pads for 30 min after injections. Mice were sacrificed two weeks after the last injections.

Brain exosome isolation: Nine months old 5xFAD mice were anesthetized using isoflurane inhalation in a chamber followed by perfusion of the whole body with cold 1x PBS using Watson-Marlow pump to remove blood- derived exosomes from the brain. Mice brains were collected, washed with 1x PBS and cut into eight sagittal slices using sterile scalpel in a petri dish. Brain slices were then transferred to C tubes containing enzymatic dissociation buffer. C tubes were tightly closed and attached upside down onto the sleeves of the gentleMACS Octo dissociator with Heaters, Program 37C_ABDK_01 being used. Samples were resuspended and applied to a MACS SmartStrainer (70 μm) placed on a 50 mL tube. 10 mL of cold D-PBS were applied onto the MACS SmartStrainer (70 μm). Cell suspensions were centrifuged at 300×g for 10 min at 4 °C, supernatants were carefully transferred to a fresh tube and supplemented with cocktail protease inhibitor (Halt™ Protease Inhibitor Cocktail, Thermo Scientific) to proceed with exosome isolation. Supernatants were centrifuged at 2000×g for 10 min followed by 10,000×g for 30–40 min then passed through a 0.45 μm filter before following the Exoeasy exosome isolation protocol following the manufacturers steps.

Tissue preparation and sectioning: Two weeks after the last 4-OHT injection and 48 hours after injection of labeled exosomes, recipient mice were euthanized, decapitated, and brains were collected. Brains were fixed in 4% paraformaldehyde overnight, then brains were moved to 15 mL conical tubes containing 30% sucrose. After the brains sank to the bottom of the tubes, they were collected, dried, and immersed in O.C.T compound in freezing molds. Bains were then slow-frozen using isopentane and liquid nitrogen. First, liquidnitrogen was put in a styrofoam box. Isopentane (Fisher Scientific, Cat#O3551-4)

was poured in a metal canister to a height of 2” and the metal canister was put into the liquid nitrogen. The two liquids were allowed to equilibrate for 10 minutes, then freezing molds containing tissues in O.C.T were immersed half-way until evenly frozen. Frozen blocks were kept in dry ice before transfer to -80 °C. Blocks were sectioned using Leica cryostat (CM1510, Leica) at temperature of -20 °C and thickness of 12-15μm, eight sections were obtained from each injected mouse around site of injection.

Immunohistochemistry: Sections were retrieved from -20 °C and allowed to dry for 15-30 min. Hydrophobic marker was used to encircle the sections, before rehydrating them in PBS. Sections were fixed with 4%PFA at 4°C for 10 min, then washed with PBS twice for 5 min. Blocking was done in 3% BSA in PBS for 30 min at room temperature, followed by primary antibody incubation for 2h at room temperature in incubation buffer (0.3% BSA in PBS) overnight at 4°C. Primary antibodies used were: anti-cleaved caspase-3 rabbit IgG (Cell Signaling, Danvers, MA, USA, #9664), anti-Tom 20 rabbit IgG (1200, Santa Cruz, sc-11415), anti-GFAP mouse IgG (1:500, abcam, Cambridge, MA, USA, ab10062). Sections were then washed 3x for 10 min with PBS, PBS-T, PBS. Secondary antibody incubation was done at room temperature for 1h in 0.3% BSA in PBS. Sections were washed 3x for 10 min each, and mounted with Fluoroshield™ with DAPI (F6057, Millipore-Sigma) or stained with NeuroTrace™ (N21479- Thermo Scientific). NeuroTrace™ was allowed to warm to room temperature, vortexed briefly, and then briefly centrifuged to deposit the DMSO at the bottom. Slides were soaked for 10 minutes in PBS + 0.1% Triton X-100, followed by two rinses in PBS, 5 min each. NeuroTrace was diluted 1:100 in PBS. 200- 250 uL were applied to each section. Slide were incubated at

room temperature for 60 min. NeuroTrace was removed and sections were washed for 10 minutes in PBS + 0.1% Triton X-100, followed by soaking in PBS twice for 5 min.

Proximity ligation assay: Tissues were prepared as mentioned earlier and incubated with primary antibodies overnight at 4°C. The primary antibodies used were: anti-Aβ mouse IgG (1:500 4G8, Biolegends, California, USA, SIG-39220), anti-VDAC1 rabbit IgG (1:1000 abcam, Cambridge, MA, USA, ab34726). Secondary PLA probes: anti-mouse MINUS affinity-purified donkey anti-mouse IgG (H + L) and anti-rabbit PLUS affinity-purified donkey anti-rabbit IgG (H + L) were diluted 1:5 in antibody diluent buffer and samples incubated for 1 h at 37 °C followed by ligation and amplification steps as described in the manufacturer's protocol (Duolink, Sigma-Aldrich). Sections were mounted using Fluoroshield supplemented with DAPI (Sigma-Aldrich) to visualize the nuclei.

3.3 Results

Firstly, labeled 5xFAD exosomes are taken up by neurons in WT mouse brain as denoted by the presence of Vybrant Cm DiI labeled exosomes inside Neurotrace positive cells (Fig. 3.1c). Interestingly, WT exosomes were taken up to a lesser extent (Fig. 3.1b). Next, we labeled brain sections for both the mitochondrial protein Tom-20 and Neurotrace to investigate the shuttling of exosomes to mitochondria as determined in our *in vitro* experiments. As expected, we found solid colocalization between 5xFAD-derived and labeled exosomes and Tom-20 inside neurons (Fig. 3.2a), which was also detectable with WT exosomes, but to a lesser extent. In order to investigate the subsequent mechanism of this interaction between mitochondria and exosomes, we performed proximity ligation assay using antibodies against Aβ and Voltage-dependent anion channel 1 (VDAC1), the main ADP/ATP transporter in the outer mitochondrial membrane. We found positive signals (denoted by green dots) inside neurons of brains injected with 5xFAD exosomes, but not in the brains injected with WT exosomes (Fig. 3.3a, b), suggesting that Aβ was shuttled via these exosomes to neurons and associated with mitochondrial VDAC1. Moreover, the association of 5xFAD exosomes and Aβ lead to caspase activation in neurons as demonstrated by the presence of cleaved-caspase signal in neurons that took up 5xFAD exosomes, which was not observed when injecting WT exosomes (Fig. 3.3c, d).

Using our astrocyte-specific exosomes reporter mouse, we show the first in-situ evidence that CD63 positive exosomes are secreted from astrocytes (Fig.3.4). Similar to the injected exosomes, these exosomes are taken up by neurons, which provide a clear evidence of communication between astrocytes and neurons via exosomes (Fig.3.5). The

astrocytes-derived exosomes also associate with mitochondria as noted by colocalization between the exosomal endogenous GFP and mitochondrial Tom-20 (Fig. 3.5). Interestingly, a subpopulation of the astrocyte-derived exosomes shuttled Aβ to neurons, confirming our *in vitro* as well as injected 5xFAD data.

In conjunction with our previous data, these results demonstrate the relevance of exosomes in Aβ induced neurotoxicity *in vivo*, suggesting that disruption of Aβ association of exosomes offers a new therapeutic approach to AD.

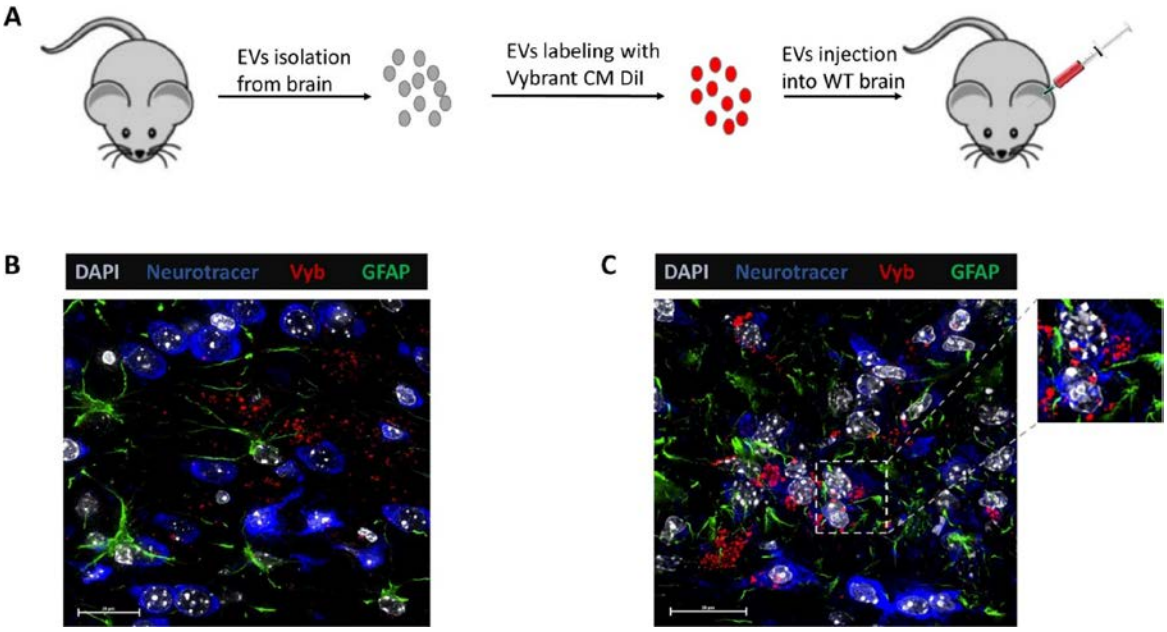

Figure 3.1: 5xFAD exosomes are taken up by WT neurons *in vivo*. (a) A schematic diagram of the experimental design. Exosomes isolated from brains of 5xFAD mice were first labeled with lipid-binding dye Vybrant CM DiI before being injected intracranially into wild type (WT) mice. Forty-eight hours post-injection, mice were sacrificed, and brains were collected and prepared for cryo-sectioning. (b, c) Representative immunocytochemistry images of sections of brains injected with (b) WT exosomes and (c) 5xFAD exosomes showing that FAD exosomes are internalized by WT neurons, insert is a higher magnification of the selected ROI

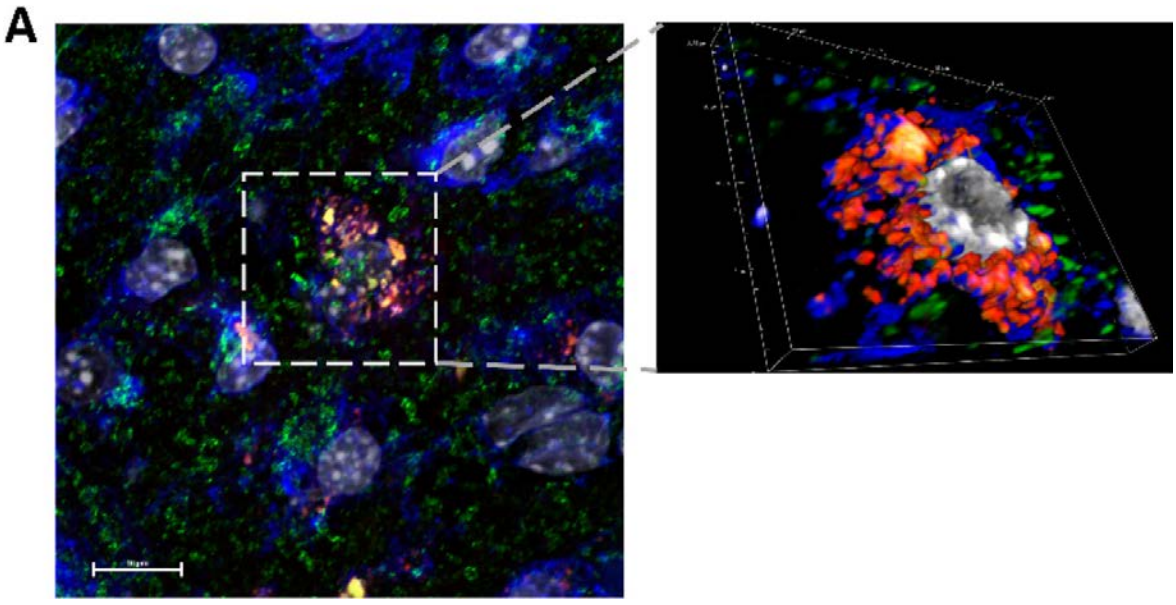

Figure 3.2: 5xFAD exosomes associate with neuronal mitochondria: (a) Representative confocal images of brain section showing that FAD exosomes (red) are colocalized with Tom-20 (green) inside neurons (blue). Insert is a 3D rendering of the selected ROI.

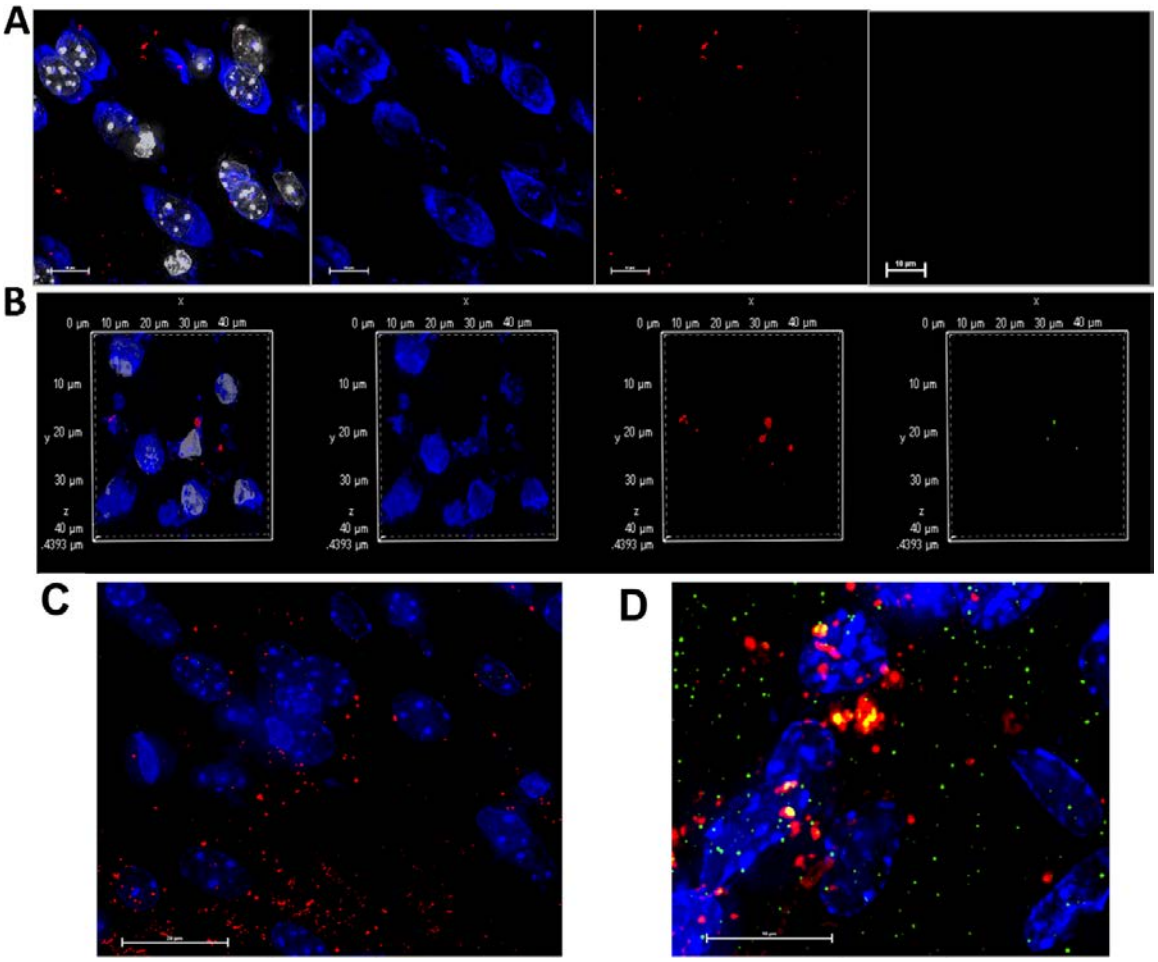

Figure 3.3: 5xFAD exosomes associate with neuronal mitochondria VDAC1 leading to caspase activation. Representative photomicrographs of (a) WT exosomes and (b) 5xFAD exosomes injected in brains of WT mice showing PLA complex formation (green) inside neurons (blue) in brains injected with 5xFAD exosomes. (c) WT exosomes injection showing no caspase activation, while (d) shows 5xFAD exosome- injected brains showing positive cleaved caspase-3 signals (green) colocalized with labeled exosomes (red).

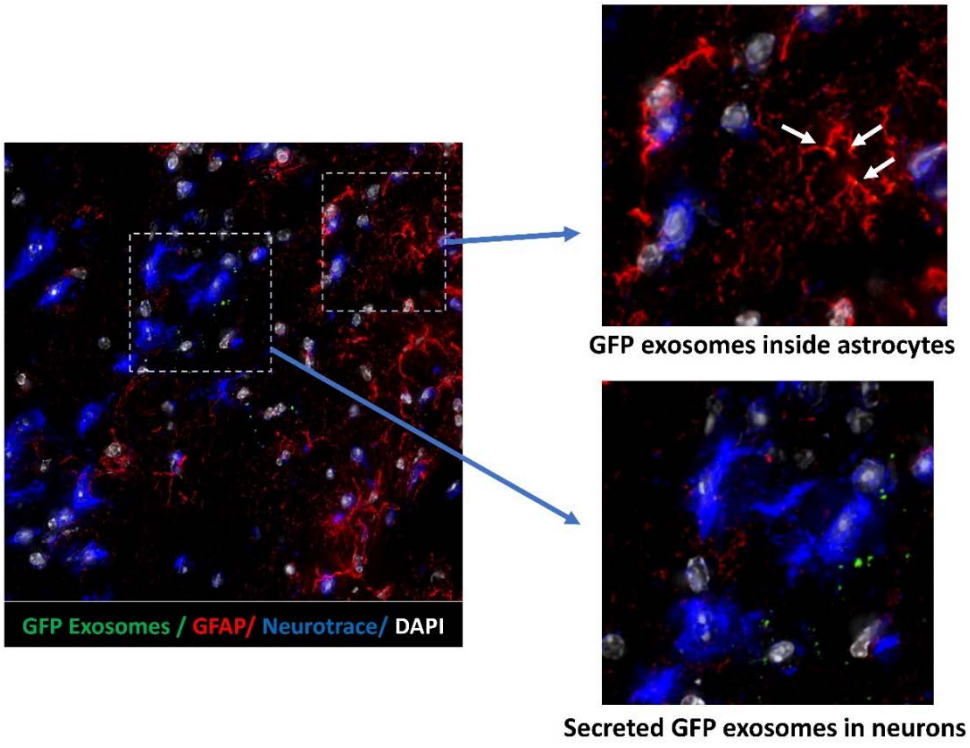

Figure 3.4: In situ illustration of astrocyte-secreted exosomes internalized into neurons. Representative confocal images of the CD63-GFP+ (green) secreted from astrocytes (red) and being taken up by nearby neurons(blue).

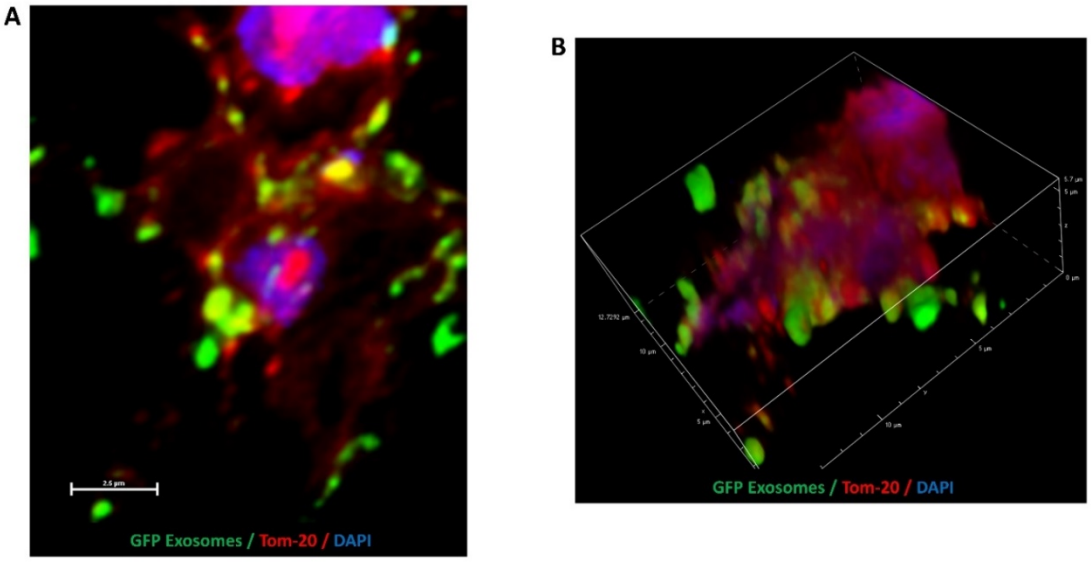

Figure 3.5: Astrocyte-secreted exosomes associate with mitochondria inside neurons. (a)Representative confocal image and 3D rendering(b) showing colocalization between the CD63-GFP+ (green) secreted from astrocytes (red) and Tom-20 (red) inside neurons.

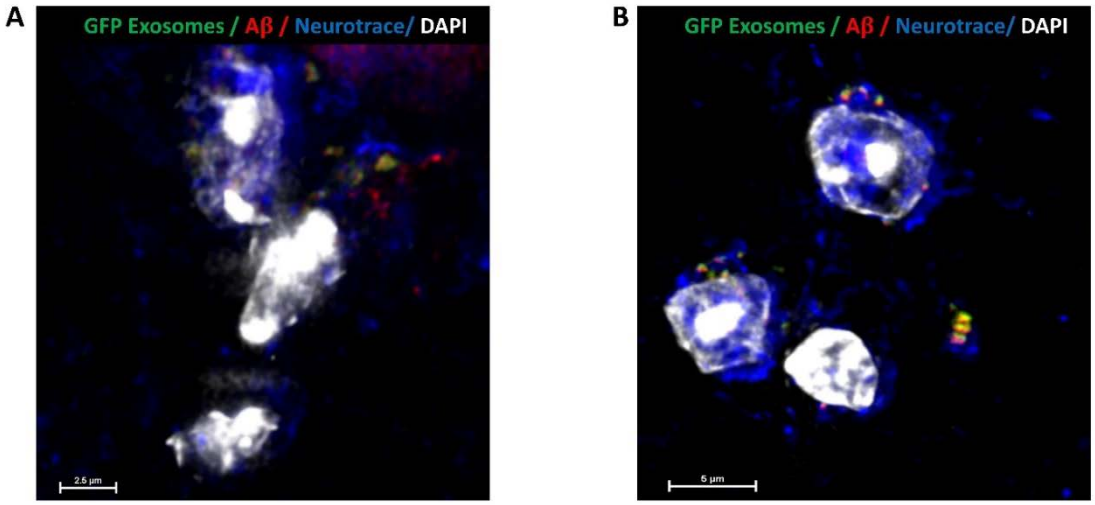

Figure 3.6: In situ evidence that astrocyte-secreted exosomes shuttle Aβ to neurons. (a)Representative confocal images showing colocalization between Aβ(red) and the astrocytes-secreted exosomes (green) in close proximity to senile plaque(a) or in plaque-free region (b).

CHAPTER 4. DISCUSSION

During the past two decades, only one drug has been approved for Alzheimer's treatment, which is the NMDA antagonist memantine[278].Numerous potential drugs and clinical trials have failed, leaving AD patients with only four valid treatments. So far, nearly all clinical trials are directed to a single aspect of the disease. These aspects include but are not limited to; fluctuations between removing or preventing aggregation of Aβ, inhibiting BACE, induction of ketosis, Serotonin reuptake inhibition and tau stabilizing. To date, none of these sole targets are able to give a complete resolution to the AD pathology. This is mainly because AD is not a simple disease with one target, but rather a complex multifactorial disease involving different cell types and numerous feedback and forward mechanisms throughout its progression, not to mention the enclosed environment where it occurs, adding an extra layer of complexity to the disease.

Astrocytes represents the most abundant glial cells in the central nervous system[279]. Under normal physiological conditions, they play key roles in brain development and function. Through their extended processes, astrocytes can contact other glial cells, synapses, and blood vessels which allow them to participate in several aspects of synapses maturation and formation, release and uptake of neurotransmitters, trophic factors production, and control of neuronal survival. Astrocytes were initially thought of as a homogenous population of cells, however, a wealth of reports indicated high levels of astrocyte's heterogenicity, both functionally and morphologically. Astrocyte's

heterogenicity could be observed localized to a certain area of the brain or generalized across brain regions[280].

There are two major subtypes of astrocytes: fibrous and protoplasmic. The fibrous astrocytes are normally prevalent within white matter and are characterized by smaller body size and less branches compared to protoplasmic astrocytes, allowing them to make contact with nodes of Ranvier aiding in maintaining homeostasis. The protoplasmic astrocytes are the most prevalent astrocytes in the grey matter. Their cell bodies are ramified, allowing them to interact with several synapses simultaneously and they are mostly found in the cerebral cortex and hippocampus[281].

In the context of AD, the likelihood of astrocytes involvement in the disease pathology was reported by Alios Alzheimer when he described the association between senile plaques and glial cells in brain tissues. However, the AD research field has focused primarily on Aβ and tau, while the glia research only gained momentum thanks to the recent GWAS discovery of several risk loci in genes related to the innate immune system, as well as the recent report implicating astrocytes and microglia in synaptic activity modulation and pruning. In AD brain samples, senile plaques are decorated with reactive astrocytes and activated microglia, however, the role of reactive astrocytes stirred controversy. Upon exposure to several stimuli, including Aβ in AD, astrocytes acquire a reactive or activatedphonotype. This activation state is manifested through hypertrophy of cellular processes, elevated GFAP expression, release of cytokines and interleukins, and increased exosomes secretion[282]. Similar to macrophages, activated astrocytes seems to be polarized into two phenotypes: A1 and A2. While A2 are viewed as protective reactive astrocytes known to secrete neuroprotective cytokines, A1 represents the neurotoxic

phenotype that lost their protective capacities and are known to upregulate a number of proinflammatory genes that have been reported to be destructive to synapses[282]. It is widely accepted that in AD and other neurodegenerative diseases that A1 astrocytes are the most abundant form, prompting researchers to study the toxic effect of those astrocytes in different diseases.

Recently, extracellular vesicles and exosomes, in particular, have emerged as contributors to the progression of disease, tools of analysis, and potential therapeutic targets[276]. Several groups reported exosomes to be transporters of Aβ, and this complex has been shown to be up taken by neurons as well as crossing the blood brain barrier. Astrocytes derived exosomes have emerged as a recent area of interest for many groups, given the crosstalk, direct and indirect interactions between different cells types in the brain, astrocytes-derived exosomes seemed a good candidate to study their participation in the disease pathology.

Within this perspective, our group was among the first to describe that reactive astrocytes' increased exosomes secretion upon exposure to Aβ. First, Wang et al. described the secretion of ceramide-rich exosomes from primary cultured astrocytes when challenged with Aβ. Not only that these exosomes were toxic to recipient astrocytes, but they also carried the prostate apoptotic protein 4 (PAR-4), however, this effect was not tested on neurons[164]. Next, astrocyte-derived exosomes were shown to promote Aβ aggregation *in vivo* and *in vitro*. This aggregation was diminished when ceramide antibody was added to the mixture. *In vivo,* chemical and genetic inhibition of nSMase2 lead to the reduction of exosome secretion, concomitant with the reduction of total Aβ and senile

plaques numbers, connecting ceramide-enriched exosomes to Aβ transfer and aggregation[226, 256].

Clearly, transferring Aβ to other cells or plaques and being toxic to recipient astrocytes are two distinct attributes of astrocyte-derived exosomes. With regards to Aβ, the previous understanding was that astrocytes-derived exosomes exert their harmful effect through inhibiting the uptake of Aβ by glial cells, leading to decreased clearance and facilitating Aβ aggregation. However, given the growing notion fueled by several reports that the size and number of amyloid plaques does not necessarily correlate with cognition deterioration in aging AD patient, we hypothesized that astrocytes-derived exosomes are mediators of the Aβ toxicity rather than merely adding them to the plaques.

In our first aim, we were concerned with the biophysical and biochemical characteristics attributed to 5xFAD exosomes compared to the WT littermates. As mentioned earlier, exosomes have the capability to freely cross the BBB. Knowing this, we used serum derived exosomes as a window to the brain.

The first difference we noticed was in the mean diameter of 5xFAD exosomes compared to the WT exosomes. While running Zetaview analysis, 5xFAD exosomes size distribution curves presented a distinct “shoulder”-containing patterns that were notobserved in the WT samples. Further clustering analysis identified an additional population in the 5xFAD exosomes with relatively higher diameter that was not present in the WT samples. The presence of this additional subpopulation means either that those exosomes are simply larger in diameter or they are more prone to aggregation. The difference in diameter lead us to question the lipid composition of the 5xFAD as a driving factor for such difference. Indeed, mass spectrometry revealed a significant enrichment in

certain ceramide species in the 5xFAD compared to the WT exosomes including C18:0, C24:0 and C24:1. The enrichment of these specific ceramide species in 5xFAD serum derived exosomes goes in line with other reports linking the higher levels of these species to several aspects of aging and neurodegenerative disease[153]. The ceramide enrichment was also observed in AD patients' serum derived exosomes compared to healthy control. While total ceramide levels were higher in both 5xFAD exosomes and AD patients' exosomes, the elevated ceramide species showed some discrepancies between 5xFAD and human samples. One explanation for that could be different levels of activity of certain ceramide synthases between the two biological systems. Since we are interested in the ceramide enriched exosomes, we used a lipid-mediated affinity chromatography (LIMAC) technique with anti-ceramide antibody in order to separate ceramide-enriched exosomes from the exosome's pools. Using that method, we showed for the first time that astrocytes derived exosomes are enriched in ceramide and carry Aβ as cargo. This supports our previous *in vitro* findings that ceramide is essential for the binding of Aβ to astrocytes-derived exosomes. Microscopy techniques using N2a cells as well as primary culture neurons, incubated with either 5xFAD and AD patients' serum exosomes, confirmed that astrocytes-derived exosomes are enriched in ceramide and shuttle Aβ into neurons.

As mentioned in chapter 1, the association between Aβ, and exosomes in general, has been reported by several groups[183, 190, 203, 208, 276, 283]. Similarly, the presence of Aβ in astrocytes-derived exosomes either *in vitro* or from AD patient's serum/plasma has previously been described as well[214, 215, 218-220]. However, the biological functions of the astrocytes-derived Aβ-harboring exosomes have not yet been clarified yet. Taking our study further, we aimed to investigate the intracellular target of our astrocytes-

derived exosomes inside neurons. One of the target organelles was mitochondria, since our group previously showed that cultured astrocytes when treated with Aβ secrete astrocytes-derived exosomes that induce apoptosis in recipient astrocytes. While studying the colocalization between astrocytes-derived exosomes and mitochondria, we noticed that 5xFAD astrocytes-derived exosomes caused mitochondrial clustering in N2a cells, this observation was absent in WT treated cells. Following up on that, we reported elevated levels of expression of DRP1, a key protein in regulating mitochondria fission. High levels of DRP1 indicated excessive mitochondrial fission, leading to mitochondria fragmentation that renders mitochondria dysfunctional. We next show that Aβ, carried on 5xFAD astrocytes-derived exosomes, directly interacts with mitochondrial VDAC1 using proximity ligation assay. This was concomitant with upregulation of cleaved caspase and subsequently induction of apoptosis, which presents a potential mechanism by which astrocytes-derived exosomes enhance the neurotoxicity of Aβ in AD.

One area of significance pertaining to this dissertation work stems from its potential to solve a huge dilemma in the AD research field while conducting Aβ-induced toxicity experiments. The exact identity of the most toxic form of Aβ is not known yet, presenting a significant knowledge gap in the field. A multitude of Aβ forms have been described in literature, and due to their heterogeneity and metastability, it is elusive which ones are relevant to AD pathology and which are simply experimental artifacts[284, 285].

This lack of information has unfavorable consequences in many areas. First, it is almost unachievable to predict which target is being engaged by antibody-directed therapeutics. Second, it is impossible to interpret data and compare results between different groups knowing that there is not a set of commonly agreed-upon experimental

conditions using the same form of toxic Aβ. The third aspect is concerning the concentration at which Aβ is used in toxicity experiments. Several studies provided different mechanisms of Aβ-influenced neuronal toxicity, however, the used Aβ concentrations are significantly higher than the reported physiologic levels of Aβ in AD patients' CSF[286, 287]. Interestingly, the astrocytes-derived exosomes - associated Aβ concentrations used in our experiments are within the femtomolar range as indicated by ELISA assay. That concentration is an order of magnitude lower than what is reported in literature, indicating a significant contribution to astrocytes-derived exosomes in sensitizing neurons to the harmful effect of Aβ. In addition, astrocytes-derived exosomes -associated Aβ provides a physiologically relevant form of the peptide that is subjected to minimal experimental alterations, if any, promoting this complex as a potential target for research and therapeutics.

The colocalization between astrocytes-derived exosomes-bound Aβ with mitochondrial marker and the direct interaction with VDAC1 raises a few questions. The first inquiry is regarding the way by which astrocytes-derived exosomes enter the cells. Several methods of exosome uptake have been reported, including simple diffusion, pinocytosis, micropinocytosis and clathrin dependent endocytosis[288, 289]. While this study did not touch on the mechanism of exosomes uptake, another group investigated that feature with respect to neuronal exosomes carrying Aβ *in vitro*. They report that the chemical drug dynasore lead to decreased uptake of exosomes and their Aβ cargo. That indicates the mechanism is dynamin dependent endocytosis, at least in SY5Y cells[190]. The next question which needs to be answered, is the mechanism by which astrocytes-derived exosomes are shuttled and directed to mitochondria specifically. As with the

uptake of exosomes, the exact route of them in a cell is not an elucidated subject yet. Several groups studied the effect on exosomes after being taken up via certain mechanisms. For example, exosomes that are taken up through phagocytosis and micropinocytosis are doomed to be cleared without having any effect on the recipient cells. However, exosomes can trigger a cellular response in case of raft-mediated endocytosis, fusion and juxtacrine or soluble signaling[290]. While that might narrow down the potential method of uptake and subsequent route of astrocytes-derived exosomes in neurons, more detailed mechanism needs to be clarified as to how these exosomes end up in direct interaction with mitochondria.

In addition, while we do show direct association between Aβ in exosomes and VDAC1, the precise mechanism is not exactly known yet. VDAC1 regulates a variety of cell functions and could be affected by a myriad of compounds, molecules and miRNAs. We show that apoptosis is the end result of the Aβ/VDAC1 interaction, however, more work needs to be done to further delineate the exact mechanism of this effect.

In that regard, ceramide may influence several aspects of our proposed mechanism. The enrichment of ceramide in the astrosomal membranes might play a role in conferring stability to these vesicles, leading to the facilitation of their binding or uptake in neurons. Also, the presence of ceramide within lipid raft might aid in the uptake process of Aβ as well. This synergestic effect of ceramide could occur by direct interaction with Aβ or facilitating its interaction with other components of the vesicle membrane, resulting in stabilizing the Aβ or inducing a specific Aβ isoforms that favor cell interaction. Moreover, ceramide might actively participate in neuronal endocytosis and transport of Aβ to mitochondria, given ceramide's ability to bind to several proteins that help directing it to

specific cellular compartment. Lastly, Due to the knowledge that ceramide interacts with VDAC1, it is conceivable that ceramide might strengthen the interaction between Aβ and VDAC1 or act as a facilitator and/or stabilizer for that binding.

Aiming to tackle some of these questions, we used a novel ceramide analog, particularly N-oleoyl serinol (S18), to interfere with the binding of Aβ to astrocytes-derived exosomes. The S18 ceramide analog reduced the average diameter of exosomes from 5xFAD mice. In addition, pre-incubation of 5xFAD exosomes with S18, prior to neuronal treatment, led to significantly lower PLA signals of VDAC1/Aβ complex. This suggests that S18 rendered the 5xFAD less prone to aggregation, judging by the decreased mean diameter of the treated vesicles. Coupled with fewer PLA signals of VDAC1/Aβ complex, these results indicate that S18 seemingly resolved the association of Aβ with astrocytes-derived exosomes.

While exosomes have been extensively studied from biological fluids, isolation of exosomes from the brain tissue is fairly recent. Levy group took the lead in establishing methods of isolating exosomes from brain tissues, a procedure that is continuously being modified as it is adopted by other groups[291]. However, those methods utilize homogenization of tissues and filtration methods that have the potential of compromising the extracellular environment with intracellular vesicles and exosome-like molecules in addition to the drawback of the after mentioned procedures being lengthy and technically challenging. In this work, we have taken a critical approach to isolation of exosomes from the brain's extracellular space. As described in section 2, we achieved that utilizing gentle dissociation of brain tissue using programmed instrumentation (GentleMax, Miltenyi Biotec) in order to avoid the presence of unwanted contaminants in our preparations. After

removing the cells, we subject the diluted extracellular fluid to a series of centrifugations followed by filtration before carrying exosome isolation using the commercially available ExoEasy kit. At this stage, we provide a new method for exosome isolation from either fresh or frozen brains, which is reliable, easy to carry out, and not time consuming. More importantly, this method preserves the integrity of exosomes and their carried cargo as confirmed via characterization of their size, morphology, and protein content.

Using this method, we isolated exosomes from 5xFAD and WT littermates' brains and repeated our experiments to study whether brain-derived exosomes would act similarly as serum derived exosomes. As expected, we found that 5xFAD exosomes showed higher levels of expression of GFAP and are significantly more toxic compared to WT ones. 5xFAD brain exosomes were shown to be apoptotic to neurons using FLICA assay, which detects early caspase activation in live cells. Interestingly, the toxicity of 5xFAD exosome was greatly mitigated when ceramide-enriched exosomes were pulled down and removed from the exosomes pool, reinforcing the idea that ceramide is essential for the harmful effect of exosomes.

Of note, we chose to use 5xFAD mouse model due to its progressive production of Aβ at early age. However, this mouse model lacks one significant physiologic hallmark of AD, namely the neurofibrillary tangles. Ptau is known to be spread via exosomes and is also known to target astrocytes, therefore a mouse model expressing ptau alongside Aβ might give a more comprehensive idea about the astrocytes-derived exosomes participation in AD pathology.

In addition, in our experiments we used total brain-derived exosomes and serum-derived exosomes from transgenic mice and AD patients. While we followed

different approaches to discern the astrocytes-derived exosomes part, it might be more helpful to use exosomes exclusively from astrocytes. While there are reported protocols to immune capture those desired exosomes from particular cell types including astrocytes, the problem relies in the yield and the starting material needed for each experiment[218]. So far, immunocapturing exosomes from a single cell type from *in vivo* origin has been used in downstream studies concerning nucleic acid determination or proteomic analysis. Functional studies require higher numbers of exosomes given the heterogeneity of the effect on neurons, which is important to be noted.

The second aim was to test the neurotoxicity of the Aβ/ astrocytes-derived exosomes complex with respect to their uptake into neuron and intracellular toxicity mechanisms, which we already touched upon in chapter 2. Taking that aim further, we studied the *in vivo* relevance of our findings that we observed *in vitro*. Labeled 5xFAD brain derived exosomes were injected into the hippocampus of two week old WT mice. 5xFAD brain derived exosomes were shown to be preferentially taken up by neurons, where they associate with mitochondrial VDAC1 and initiate apoptosis. This data significantly corroborates the *in vitro* studies, however it does not delineate the origin of the up taken exosomes.

Aiming to visualize the endogenous exosome transfer from astrocytes to neurons, we generated a triple transgenic mouse model that secretes GFP-labeled exosomes exclusively from astrocytes, which allows us to track them and their uptake. As with the injected exosomes, we found GFP-labeled exosomes inside nearby neurons and around amyloid plaques. We also found that they associate with amyloid beta and colocalize with mitochondria. To our knowledge, this work provides the first *in vivo* evidence of the

transport of Aβ from astrocytes to neurons via exosome, adding a new layer of complexity to the AD pathology given the neuroprotective roles attributed to astrocytes in normal conditions. The significance of these finding relies on the facts the astrocytes are the most abundant cell types in the brain. Even the slightest contribution to the spreading of the most toxic form of Aβ could significantly exacerbate the pathology of the disease. This finding is in accordance with new reports implicating astrocytes in the metabolism and generation of Aβ.

Questions may arise concerning the origin of the Aβ associated exosomes. While we focus on astrocytes derived exosomes in this work, it is not to be excluded that other cell types in the brain secrete exosomes that can associate with Aβ. In fact, most of the publication regarding Aβ association with exosomes are done in neuronal cultures. Similar to the distinct cargo depending on the originating cell type, the fate of exosomes and their uptake by different cell types seems to be governed by their donor cells. For instance, microglia derived exosomes are thought to play a protective role when it comes to Aβ handling[292]. Exosomes from microglia mainly bind to Aβ and aid in the clearance of the peptide, preventing its uptake by neurons. In addition, the specificity of astrocytes-derived exosomes uptake by neurons has been independently shown by separate groups utilizing *in vitro* models[293]. That prompted us to build on our group's previous work, proposing astrocytes-derived exosomes as the main toxic exosomes in the brain.

In conclusion, irrespective of the inherent limitations of our study, our data advocate that the Aβ/ astrocytes-derived exosomes association is a viable target for treatment of AD to suppress the Aβ-induced toxicity.

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VITA

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1. EDUCATION

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2. RESEARCH AND INTELLECTUAL CONTRIBUTIONS

A. Publications

- **Ahmed Elsherbini**, Haiyan Qin, Zhihui Zhu, Priyanka Tripathi, Simone M. Crivelli, and Erhard Bieberich. In vivo evidence of exosome-mediated Aβ neurotoxicity. Acta Neuropathologica communication 8, Article number: 100 (2020)

- **Ahmed Elsherbini**, Alexander S. Kirov, Michael B. Dinkins, Guanghu Wang, Haiyan Qin, Zhihui Zhu, Priyanka Tripathi, Simone M. Crivelli, and Erhard Bieberich. Association of Aβ with ceramide-enriched astrosomes mediates Aβ neurotoxicity. Acta Neuropathologica communication volume 8, Article number: 60 (2020).

- **Ahmed Elsherbini**, Haiyan Qin, Zhihui Zhu, Priyanka Tripathi, Guanghu Wang, Simone M. Crivelli, Stefanka D. Spassieva, and Erhard Bieberich. Extracellular Vesicles Containing Ceramide-Rich Platforms: "Mobile Raft" Isolation and Analysis. Methods in Molecular Biology, accepted.

- Priyanka Tripathi, Zhihui Zhu, Haiyan Qin, **Ahmed Elsherbini**, Emily A. Roush, Simone M. Crivelli, Stefanka D. Spassieva, and Erhard Bieberich. Cross-Link/Proximity Ligation Assay for Visualization of Lipid and Protein

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B. Local, National, and International Symposia

- **Ahmed Elsherbini**, Alexander Kirov, Michael B. Dinkins, Sanjib Karki, Simone M. Crivelli, Haiyan Qin, Zhihui Zhu, Priyanka Tripathi, and Erhard Bieberich. "*Astrocyte-derived ceramide- enriched exosomes ("astrosomes") enhance neurotoxicity of Amyloid beta*" **Southeastern Regional Lipid Conference Ashville, Nov 2019**

- **Ahmed Elsherbini**, Alexander Kirov, Michael B. Dinkins, Sanjib Karki, Simone M. Crivelli, Haiyan Qin, Zhihui Zhu, Priyanka Tripathi, and Erhard Bieberich. *"Association of Aβ with astrocyte-derived and ceramide- enriched exosomes mediates Aβ mitotoxicity in neurons which is prevented by novel ceramide analogs"* **Society for Neuroscience. Chicago, Oct 2019**

- **Elsherbini A**, Dinkins M, Leanhart S, Zhong L, Wang G, Jiang X, Zhu Z, Spassieva S, Qin H, and Bieberich E. "*Astrocyte-derived ceramide- enriched exosomes ("astrosomes") enhance neurotoxicity of Amyloid beta*" **Southeastern Regional Lipid Conference- Cashiers, Sep2018**

- **A. Elsherbini**, A. Kirov, M. Dinkins, S. Leanhart, L. Zhong, G. Wang1, S. Spassieva, M. Sauer, A. Schubert-Unkmeir, E. Bieberich."*Characterization of molecular interaction between exosomal ceramide and beta amyloid, revealing a role of ceramide in Abeta aggregation and toxicity"* ***Society for Neuroscience. San - Diego, September 2018***

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- **Ahmed Elsherbini**, Alexander Kirov , Michael Dinkins, Silvia Leanhart , Guanghu Wang, Haiyan Qin , Anne Burgert, Markus Sauer , Alexandra Schubert-Unkmeir, Zhihui Zhu, Stephanka Spassieva , Xue jiang , Lianshing Zhong, Erhard Bieberich." *Characterization of molecular interaction between exosomal ceramide and beta amyloid"*

- Jessica L. Pierce, Kanglun Yu, **Ahmed Elsherbini**, Elizabeth W. Bradley, Jennifer J. Westendorf, Meghan E. McGee-Lawrence. *Osteoblastic Hdac3 Expression Regulates Systemic Energy Metabolism.* ***American Society for Neurochemistry. Riverside, March2018***

- Jason Conger, Matt Manning, **Ahmed Elsherbini**, Brendan ware, Behren Bass, Patricia Schoenlein. *Targeting MEK/MAPK1/2 In- Vivo to Eradicate Antiestrogen Resistance.* ***The American Society for Bone and Mineral Research. Atlanta, September 2016***

- Emily Bass, Matt Manning, **Ahmed Elsherbini**, Brendan Ware, Meghan McGee-Lawrence, Patricia Schoenlein. *HDAC targeting to potentially optimize BimEL-induced apoptosis in antiestrogen- treated breast cancer cells.* ***Medical Scholars Research Day. Augusta, September 2015***

- Sadanand Fulzele, **Ahmed Elsherbini**, Saif Ahmad, Rajini Sangani, Suraporn Matragoon, Azza El-Remessy, Gregory I. Liou. The microRNA miR-146b-3p controls diabetic retinal inflammation by suppressing adenosine deaminase-2. **The Association for Research in Vision and Ophthalmology (ARVO) annual meeting, Seattle, May2014**

3. Talks

- *"Ceramide-enriched exosomes enhances neurotoxicity of Aβ in Alzheimer's disease"* ***International Society of Extracellular vesicle, Virtual, August 2020.***

- **"***Association of Abeta with astrocytes-derived and ceramide-enriched exosomes mediates Abeta neurotoxicity"* ***Southeastern Regional Lipid Conference. Ashville, November 2019.***

- *" Characterization of the molecular interaction between exosomal ceramide and beta amyloid, revealing a role of ceramide in Abeta aggregation and toxicity."* **American Society for Exosomes and Macrovesicles. Baltimore, October 2018.**

- *"Astrocyte-derived ceramide-enriched exosomes ("astrosomes") enhance neurotoxicity of Amyloid beta"* **Southeastern Regional Lipid Conference. Cashiers, Sep2018.**

4. Awards

- PhD contest finalist***, Sphingolipids Biology Seminar Series. Virtual, October 2020.***
- Travel award, ***Southeastern Regional Lipid Conference. Ashville, November 2019.***
- Travel award, ***Southeastern Regional Lipid Conference. Cashiers, Sep2018***