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Analysis of the role of the neuronal receptor LRP8 in the production of exosomes in a cellular model and in the context of Alzheimer's disease

Tutor: **PhD Student:**

Prof. Claudio RUSSO **Donatella MIGNOGNA**

Prof. Ferdinando CARANCI **Matr.160521**

Coordinator:

Prof. Marco Sarchiapone

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ABSTRACT

An increasing number of evidences has revealed that alterations of endosomal–lysosomal system are connected to alterated sorting and trafficking of different proteins, leading to a significant alteration of protein homeostasis itself. These alterations result particularly compromising in the field of neurodegenerative diseases [1].

In this scenario, exosomes, extracellular vesicles derived from endosomal–lysosomal system, reflecting the alterations present in the cells that produced them, have attracted the attention of the scientific community, as possible early markers of disease. In the field of Alzheimer Disease, exosomes have been shown the capacity to reduce brain Amyloid-beta involving microglial uptake, and negative effects as spreading hyperphosphorylated tau, therefore they could be involved in the mechanisms of apoptosis and, ultimately, contribute to dendritic degeneration [2].

Apolipoprotein E4 expression, the most relevant factor in Late Onset Alzheimer Disease, has a negative impact on exosomes production, both in human brain derived exosomes, and in humanized mouse model expressing ApoE4 allele [3]. One of the target of Apolipoprotein E is Low-density lipoprotein Receptor-related Protein 8 (LRP8 or ApoER2), highly expressed in neuronal tissue and actively involved in memory formation and spines dendridic modeling [4].

Considering that we previously observed that LRP8 localization and processing are alterated in the cerebral cortex of sporadic and familial

Alzheimer's Disease patients, we decided to analyze the role LRP8 on exosomes production.

We found that in exosomes derived from brain of patients affected by sporadic and familial Alzheimer (SAD and FAD), C-terminal fragments of the LRP8 receptor with molecular weight less than 15 kDa are strongly present, which instead are not evident in controls; a sign that the proteolytic processing of LRP8 is strongly altered in case of disease and that the contents of the vesicles change radically. The production of exosomes also changes radically, being strongly compromised in the case of FAD. We performed in-vitro experiments using Neuro 2A wild-type cells, stably transfected with human LRP8 (hLRP8) receptor (Neuro 2A DDK myc and Neuro 2A LRP8 HA), and we found that LRP8 receptor expression significantly increases exosomes production. We also expressed in Neuro 2A wild-type cells, the human protein Amyloid Precursor Protein 695 (hAPP 695), and we observed that LRP8 C-terminal fragments are present both in the cell lysates and in the exosomes. We also reported that recombinant human ApoE4 and DAPT (a γ-Secretase inihibitor) treatment, decrease exosomes production in-vitro, and that ApoE4 treatment increase LRP8 C-terminal fragments in exosomes. Finally, to confirm that LRP8 receptor is involved in exosomes production, we performed a silencing on the LRP8 receptor using the Neuro 2A wild-type cells. We observed that silencing the expression of LRP8, there is a significant reduction in the number of exosomes produced.

INTRODUCTION

A feature common to many neurodegenerative diseases is the progressive accumulation of protein aggregates inside and outside the neurons, in defined brain regions, which ultimately are responsible for the clinical phenotype of the disease. In most cases, these complex can be formed from the mutant form of a particular protein, which by aggregating or folding abnormally loses its natural solubility and becomes highly cytotoxic [5]. These series of events are considered a kind of biochemical signature of a significant alteration in protein homeostasis.

Among the systems that deal with maintaining the integrity of a functional proteome, the efficient sorting and trafficking of different proteins, the endosomal-lysosomal system plays a crucial role. From this system derive the exosomes, extracellular vesicles, which in recent years have attracted a lot of attention from the scientific community, in particular for the study of neurodegenerative diseases and, in this specific case, Alzheimer's disease (AD), in order to identify new therapeutic and clinical goals.

AD is the most common form of dementia and may contribute to 60–70% of cases worldwide [6]. In 2020 Word Health Organization described Dementia as a "syndrome of a chronic or progressive nature in which there is deterioration in cognitive function beyond what might be expected from normal ageing. It affects memory, thinking, orientation, comprehension, calculation, learning capacity, language, and judgement. The impairment in cognitive functions are commonly accompanied, and occasionally preceded, by deterioration in emotional control, social behaviour, or motivation".

Brain changes associated with Alzheimer's Disease

Two of several brain changes associated with AD are tau tangles and beta-Amyloid plaques, inside and outside neuron, respectively [7]. Tau tangles are the result of an increased phosphorylation and aggregation of the protein tau, instead, the accumulation of the protein fragments β-Amyloid in β-Amyloid plaques derived from a proteolitc processing of Amyloid Precursor Protein (APP), a primarly protein involved in AD. Tau tangles inhibit the transport of nutrients and other essential molecules inside neurons and plaques can interfere with neuron-neuron communication at the synaptic level and contribute to the damage and death of neurons (neurodegeneration) [8, 9].

Other brain changes in AD, include atrophy and inflammation. The accumulation of toxic beta-Amyloid and hyperphosphorylated tau, are able to activate resident immune system cells in the brain, microglia and astrocytes, leading to a release of citokine, chemochine, caspaseses, and activating the complement system, a major constituent of the innate immune system, mainly involved in defence against pathogens. All these are factors that drive neuroinflammation [10].

Atrophy in the brain affects especially hippocampus, and occurs primarily because of cell loss [11].

Physiological functions of the brain are further impaired in AD by the decrease in the brain's ability to metabolize glucose. [12].

Stages of Alzheimer Disease

The disease is divided into several stages: Preclinical AD, Mild Cognitive Impairment (MCI), Mild, Moderate and Severe AD.

In the first stage, even if the individual does not shows symptoms such as memory loss, some brain changes will be measurable. Decreased metabolism of glucose as shown on PET scans and abnormal levels of beta-Amyloid on positron emission tomography (PET) scans and in analysis of cerebrospinal fluid (CSF), are generally biomarkers for AD.

Not all individuals with evidence of Alzheimer's-related brain changes develop symptoms related to Alzheimer's. For example, some individuals have beta-Amyloid plaques at death but have had no memory problems throughout their lives [13].

In the MCI due to AD, are present evidences of impairment in one or more cognitive domains, typically including memory, that reflect a change in cognition, reported by patient or clinician and preservation of independence in functional abilities [14].

In the moderate stage of Alzheimer's dementia, which is often the longest stage, individuals may have difficulty communicating and carrying out activities of daily living (such as dressing or bathing), and may begin to exhibit behavioral changes.

In the final stages of the disease, the patient shows severe impairment of all cognitive functions, inability to recognize family members, physical impairment generalized and inability to perform basic activities of daily living [15].

AD is often thought of as a purely mental illness, but this is not the case; in fact, the effects on physical health deriving from brain damage are manifested above all in the final phase of the disease.

In the severe stage of Alzheimer's dementia, people need help with activities of daily living and are likely to require 24 hour assistance. Due to damage to the areas of the brain involved in movement, people are left bedridden. Being bedridden makes them vulnerable to conditions such as skin infections and sepsis and blood clot formation.

Damage to the areas of the brain that control swallowing make it difficult to eat and drink. This can cause food to be swallowed into the trachea instead of the esophagus. Because of this, food particles can settle in the lungs and cause lung infections. This type of infection is called aspiration pneumonia and causes death among many individuals with Alzheimer's [16].

Diagnosis of AD

- There is currently no single test to diagnose dementia due to Alzheimer's disease. Often, therefore, a variety of approaches and tools are used to help make a diagnosis and they include the following:
- Ask a family member to provide informations on changes in thinking skills and behavior
- Reconstruct the individual's medical and family history, including psychiatric history and the history of cognitive and behavioral changes manifested
- Administer cognitive tests, physical and neurological exams
- Have the person undergo blood tests and brain imaging to rule out other potential causes of dementia symptoms
- In some cases, cerebrospinal fluid (CSF) analysis to determine levels of beta-Amyloid and certain types of tau

It is clear, therefore, that it is almost always possible to determine whether a person has dementia, but at the same time it can be difficult to identify the exact cause [17].

Due to the difficulty in diagnosing Alzheimer's dementia, currently one of the greatest challenges for the scientific community in this field, is to find a way to make a certain diagnosis of the disease, based on early and specific markers.

Risk factors for Alzheimer Disease

In most cases, Alzheimer's dementia occurs around the age of 65 or older, in these cases we speak of late onset Alzheimer's disease (LOAD). In some other cases AD can affect middle-aged subjects, in these cases we speak of early onset Alzheimer's disease (EOAD); the earliest documented case it's about a 28-year-old man [18].

When the onset of the disease is so early, the patient is very likely to have Familial Alzheimer's Disease (FAD), and the genetic causes are known. However, when onset is late and no genetic causes are identifiable, we are faced with sporadic Alzheimer's disease (SAD).

Genetic mutations

A small percentage of Alzheimer's cases [19] develop as a result of mutations in any of three specific genes. Mutations within the gene on chromosome 21, encoding for APP, the precursor of beta-Amyloid peptides, are associated with 2-3% of all EOAD cases.

Mutations in the gene encoding for presenilin 1 (PS1), located on chromosome 14 and in the gene encoding for presenilin 2 (PS2), located on chromosome 1, are linked to 70% and 20 % of the EOAD cases. Presenilins are components of γ-Secretase complex, which, among the various substrates, also cuts APP. Mutations in these 3 genes (APP, PS1 and PS2) alter the processing of APP, leading to an increase in the production of beta-Amyloid [20].

Not all known mutations in the gene encoding for APP are associated with AD, indeed, some are thought to be protective against the disease [21].

According to the "amyloid cascade hypothesis", the best known theory on the etiology of AD, however, deposition of beta-Amyloid represents a primary event in the pathogenesis of AD, followed by all other features of the disease [22].

Trisomy in Down syndrome

People with Down syndrome have a higher risk of developing AD, and this is thought to be related to trisomy 21. Having an extra copy of chromosome 21 means, in fact, increase the production of beta-Amyloid fragments. From the age of 40, most people with Down syndrome show significant levels of beta-Amyloid plaques and tau tangles in the brain [23].

Trem 2

TREM2 belongs to a family of receptors called Trigger Receptors Expressed on Myeloid cells (TREM) [24]. In the brain, TREM2 is strongly expressed by microglia [25], mainly in the spinal cord, hippocampus and white matter [26].

TREM2 expression has been observed to be impaired in pathological conditions such as amyotrophic lateral sclerosis (ALS) [27], traumatic brain injury [28], Parkinson's disease [29] and AD [30], in which increased activation of microglia has been observed around the beta-Amyloid plaques [31]. Aging is also a factor that increases TREM2 expression both in mice and humans [32].

In AD has been hypothesized that the chronic inflammation observed in the brain results in an increase in TREM2. In recent years, thanks to the development of whole genome sequencing and genome wide association studies (GWAS), several genetic variants of TREM2 have been identified that increase the risk of developing LOAD [33].

Among them, several variants significantly increase the risk of LOAD by 2 to 4 times, comparable to the risk of LOAD associated with APOE4 allele.

In 2013, individuals heterozygous for the rare R47H variant of TREM2 were found to have a higher risk of developing AD than individuals who do not carry this variant [34].

The role of TREM in the pathogenesis of AD remains controversial because some studies suggest that TREM2 activity during the early stages of AD affects the formation of beta-Amyloid containing plaques, while other studies show that TREM2 expression in a tau model of AD is more damaging during disease progression [35].

The Apolipoprotein E4

One of the main factors in the maintenance of synaptic integrity and plasticity, is Apolipoprotein E mediated transport of cholesterol from astrocytes to neurons [36;37].

In humans there are three main alleles of the apolipoprotein E (APOE) gene: APOE2, APOE3 and APOE4, that encode for Apolipoprotein E2, Apolipoprotein E3 and Apolipoprotein E4, with an approximate worldwide distribution of 7%, 79% and 14%, respectively [38].

The expression of APOE4 allelic variant has strong consequences on the brain, including an increased risk of LOAD [39].

Carriers of the APOE4 allelic variant have an increased risk [40; 41] 3-4 times and 9-15 times in heterozygosity and homozygosity, respectively to develop AD [42; 43] compared to the more common APOE3 carriers.

Generally the APOE2 isoform is associated with a reduced risk [44; 45] to develop this dementia. Given that individuals suffering from LOAD represent more than 95% of the total AD population, it is crucial to clarify the link between ApoE4 and the signs of the disease. The expression of ApoE4 has effects on tau phosphorylation, tau-mediated neurodegeneration [46], and the formation of beta-Amyloid plaques; ApoE4, infact, has a role on the deposition and clearance of beta-Amyloid, because it interacts directly with the protein [47].

The story of the most known theory on the origin of Alzheimer: Amyloid Hypothesis

Since Alois Alzheimer first illustrated the cognitive decline of a woman named Auguste D, in 1906 [48], and he observed that extracellular plaques of beta-Amyloid were present in the brain [49; 50], beta-Amyloid plaques became a histological sign of AD. In the years that followed, the scientific community focused on understanding AD, probably caused by beta-Amyloid.

When beta-Amyloid protein is processed from APP by α-Secretase and BACE (β-Secretase) [51], the N-terminal fragments generated by this initial cleavage are called sAPP-α and sAPP-β, respectively, while the C-terminal fragments generated by α-Secretase and BACE cleavage are called C83 and C99, respectively. In both cases, the C-terminal fragments undergoes a further cut by the γ-Secretase enzyme complex (composed, in addition to PS 1 and 2, of Nicastrin, and Enhancer Anterior Pharynx Defective 1) (**fig. 1**).

From this processing derive a fragment called AICD (APP intracellular domain) and the p3 peptide in the case of the α-Secretase pathway or the beta-Amyloid protein in the case of the BACE pathway.

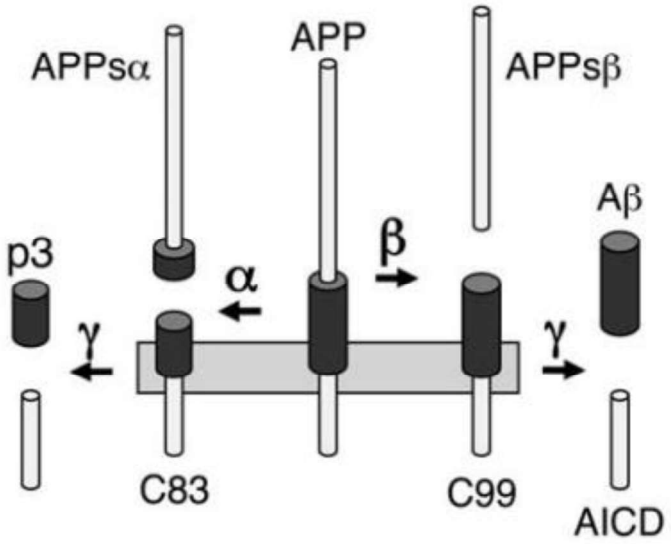

Figure 1– Schematic view of APP processing by α, β and γ Secretase. Adapted from Lichtenthaler 2012.

Simultaneously with the discovery of beta-Amyloid as a major component of amyloid plaques in AD, the earliest genetic causes of the disease have been identified. Despite only 1-6% of AD cases are caused by mutations, the discovery of these rare cases generated great excitement, because it was believed that studying the families in which these mutations were present would shed light on the pathogenesis of the disease in all cases. After various studies it has become clear that these mutations affect the way in which APP is processed, resulting in an increase in beta-Amyloid production [52; 53].

Essentially, these were the findings that fueled the hypothesis of the Amyloid theory, according to which, beta-Amyloid deposition is a central event in the etiology of AD [22].

Originally, this hypothesis also suggested that beta-Amyloid deposition led to tau pathology, resulting in cell death and that understanding this cascade of events would aid rational drug design [22]. This view was not only consistent with known familial cases of AD, but also integrated with the observation that patients with Down syndrome who lived long enough, developed AD [54]. It was subsequently shown that the beta-Amyloid levels found in AD are toxic to neurons [55, 56].

While the neurofibrillary tangles of the tau protein are the other sign of AD, the Amyloid hypothesis has marginalized the tau-centric view of AD. This is also because pathogenic tau mutations are responsible for neurodegeneration, but do not cause AD. More clearly, mutations in tau cause neurodegeneration with the presence of neurofibrillary tangles, but without beta-Amyloid plaques [57], while mutations in APP cause neurodegeneration with beta-Amyloid plaques and neurofibrillary tangles containing tau, identical to the features found in SAD patients [19]. So, it turned out that the AD had to be caused by β-amyloid deposits [22].

The crisis of the Amyloid Hypothesis

Initially, the idea was to develop drugs that inhibit γ-Secretase, in order to, ultimately, reduce beta-Amyloid production. This approach seemed promising, also considering the difficulties in the development of BACE inhibitors [58].

One of the best known inhibitors of γ-Secretase is Semagacestat [59]. It has been shown that the Semagacestat was capable of reduce the production of beta-Amyloid in the brain, in healthy volunteers [60], but, subsequently, two phase III studies were interrupted due to failure to demonstrate efficacy. Patients who received Semagacestat worsened cognitively, and more importantly, they showed an increased risk of develop skin cancer, compared to those who took placebo [61].

Another therapeutic approach based on the Amyloid hypothesis focused on inhibiting the formation of beta-Amyloid oligomers. Some studies suggested that the neurotoxic form of beta-Amyloid consists of soluble oligomers instead of monomers [62; 63; 64].

Therefore, drugs such as Tramiprosate were studied. In preclinical studies, Tramiprosate reduced levels of beta-Amyloide both in the mouse brain and in plasma, thereby preventing plaques formation [65]. However, in a phase III study, patients with AD showed no significant overall results [66].

The most recent strategy adopted is based on the elimination of beta-Amyloid by the immune system. This approach can be active in case of administration of an epitope that generates an immune response or it can be passive in case of antibodies directed against beta-Amyloid. The active immunization approach was immediately abandoned following a clinical study in which meningoencephalitis was found in vaccinated patients [67].

Regarding passive immunization, the most discussed monoclonal antibodies directed against beta-Amyloid were Bapineuzumab and Solanezumab. Regarding the former, Pfizer and Johnson & Johnson announced its failure during a phase III trial in 2012, [68]. As for Solanezumab, Eli Lilly announced the failure of phase 3 in 2016 [69].

In recent decades, therefore, increasing evidence has shown that the increase in beta-Amyloid levels in the brain may not be a key phenomenon in the pathogenesis of AD, leading to the need to identify new clinical and therapeutic targets.

Very interesting, in this scenario, is the recent case of identification of a PSEN1 mutation carrier who developed very mild cognitive impairment up to the age of seventy, many years after the classic onset of EOAD. This patient had two copies of the Christchurch APOE3 mutation (R136S), very high levels of beta-Amyloid and limited phosphorylated tau and neurodegeneration [70].

This case stresses once again that the accumulation of beta-Amyloid in extracellular plaques is likely a manifestation of the disease but not a primary cause and that it is essential to identify new therapeutic and clinical targets for Alzheimer's disease.

Exosomes: biogenesis, biologic function and clinical potential

It's know that exosomes, nano-sized vesicles (50 to 150 nm diameter), released from many cell types, are implicated in AD and that they could be able to carry pathogenic proteins and contribute to spreading of the disease in the brain [71].

In general, they are able to carry cell-specific cargos of lipids, proteins and genetic materials, and can be selectively taken up by neighboring or distant cells far from their release.

Exosome biogenesis starts inside of the endosomal system: early endosomes mature into late endosomes, and subsequently in Multi-Vesicular Bodies (MVBs). During this process endosomal membrane invaginates to generate intra-luminal vesicles (ILVs) in the lumen of the organelle [72; 73]. MVBs can fuse with lysosomes to degrade their content, or they can fuse with the plasma membrane to release ILVs into the extracellular space [74; 75]. The most characterized pathway for the formation of ILVs and MVBs is driven by The Endosomal Sorting Complex Required for Transport (ESCRT) machinery and it is composed by different proteins assembled into four complexes (numbered from ESCRT 0 to 3) with associated some proteins as ALIX, commonly used as exosomes markers [76]. ESCRT machinery recognizes the ubiquitylated proteins: ESCRT 0 recruits proteins for internalization, ESCRT 1 and 2 are involved into the budding process and promote formation of MVBs and ILVs, allowing cytosolic proteins to enter during biogenesis of exosomes, finally, ESCRT 3 complex is involved in membrane invagination and separation [77]. The trafficking of MVBs throughout the membrane or toward lysosomes is regulated by a GTPase proteins, belonging to the Rab family [78].

Exosomes and their cargos may offer diagnostic information in a range of diseases, such as chronic inflammation [79], renal and cardiovascular diseases [80], neurodegenerative diseases [81] and tumors [82].

Regarding tumors, for example, exosomes are involved in cancer progression and in the metastatic cascade. The exosomes derived from cancer cells can participate in metastatic dissemination of a primary tumor, promoting a series of biological processes including resistance to apoptosis, proliferation, and angiogenesis. [83; 84]. It was also shown that exosomes derived from the cancer cells induce death of the immune cells inhibit their activation or their differentiation [85]. As for the processes of metastasis, also for Alzheimer's disease, cellular communication is very important. We know, in fact, that the progression of the disease, at the cerebal level, involves in sequence specific areas that show an increase in beta-Amyloid and hyperphosphorylated tau over time. This means that intercellular communication in AD is not causal, and that extracellular vesicles, such as exosomes, may play a fundamental role in conveying pathogenic information. Considering the complexity of AD disease, the many failures in identifying the cause of AD in the Amyloid theory, and in the light of what has been said, we decided to analyze the role that exosomes can have in Alzheimer's disease, focusing on LRP8, as a possible new marker of disease.

Lipoprotein Receptor-related Protein 8 (LRP8): a link between APP, Gamma Secretase, ApoE

We have chosen to focus our attention on the neuronal LRP8 receptor, for a variety of reasons. Initially, our choice was to identify new proteins, which shared some aspects with APP.

These proteins had to be, highly expressed at the neuronal level, a substrate of γ-Secretase, involved in memory processes and targets of ApoE. Our choice, therefore, fell on the LRP8 receptor, belonging to the LDL receptor family.

The receptors belonging to this family are involved in a multitude of cell signaling pathways [86], and many of these receptors interact with APP; LRP8, for example, shares with APP, cytosolic signaling adapter proteins such as X11/Mint, Disabled-1 (Dab1), FE65 and it is target of ApoE [86].

Regarding the interactions with APP we know that the integrity of LRP8 intracellular domain, NPXY, involved in the mechanisms of internalization of the receptor itself, influences the interaction with APP and, ultimately, the formation of beta-Amyloid [87]. On the other hand, it has been shown that the interaction of LRP8 with some of its ligands such as F-spondin, induced LRP8 inhibition of APP endocytosis and, ultimately, a decrease in Amyloid production [88]. As regards the link between LRP8 and ApoE, it has been observed that this interaction triggers the endocytosis of APP with the enzyme BACE, favoring the production of β-Amyloid [89], in particular in the presence of ApoE4.

From these observations we can understand how the consequences of the binding between APP and LRP8 are not simple and unique.

Finally, it has also been shown that the interaction of LRP8 with Reelin, a glycoprotein representing another important LRP8 ligand, stabilizes the interaction with the intracellular adapter protein Dab1, both with LRP8 and APP, and this interaction ultimately leads to a lower production of beta-Amyloid [90].

The extracellular protein Reelin is fundamental for neuronal migration during brain development [91]. Reelin also exerts another function in the mature brain: it is able to modulate synaptic plasticity playing a role in the formation of long-term memory.

This action is mainly carried out thanks to the interaction with the LRP8 receptor. In this process, a particular importance is once again played by the intracellular domain of LRP8, which promotes the binding and phosphorylation of NMDA receptors, leading to an increase in long-term potentiation [92]. To understand more thoroughly how important the functions associated with the LRP8 receptor and the links with the proteins with which it interacts are, we just think that the LRP8 knock-out (KO) mice show deficits in long-term potentiation, suggesting a role of this receptor in the synaptic plasticity of the adult brain [93] and Reelin knock-out mice also show the same phenotype, well known as the Reeler phenotype, characterized by the inversion of cortical layers and the absence of cerebellar foliation [93]. Reelin, also promotes LRP8 proteolytic processing, with the release of the intracellular domain (ICD) LRP8, which regulates the expression of Reelin itself [94].

AIM OF THE STUDY

Recent studies have shown that there is a decrease in LRP8 expression during aging, and this is linked to cognitive decline in mice [95], underlining the importance of the LRP8 receptor in memory processes, and in memory dysfunction diseases.

Although the proteolytic processing of APP has been deeply analyzed over time, much less is known about the proteolytic processing of the LRP8 receptor, despite the centrality of the receptor in memory processes.

For this reason, in the past we decided to produce an antibody directed against a specific sequence present at the C-terminus of LRP8. Analyzing human brain, CSF and plasma samples deriving from both SAD and FAD patients, we observed that LRP8 undergoes an abnormal processing in case of disease, which results in a significant increase in low molecular weight fragments, ranging from 12 to 8 kDa; these fragments were named LICDs (LRP8 INTRACELLULAR DOMAINS).

Our previous in-vitro studies have shown that even in the absence or blockade of the γ- Secretase complex, these fragments are equally present, an observation that led us to believe that other proteases were involved in the proteolytic processing of LRP8. Some fascinating question marks have come up at this point, such as "What is the significance of these fragments? Where can they be found? Do they have extracellular or intracellular activity? What affects them?"

Considering the recent interest in exosomes and their role as a possible clinical and therapeutic target in the vast field of dementias, we have chosen to investigate a possible relationship between LRP8 receptor and exosomes production, starting from ex-vivo human brain tissues. We analyzed the

processing of the LRP8 receptor, in exosomes deriving from ex-vivo human brain tissues, both in SAD and FAD conditions, in order to analyze variations in the receptor processing, similar to what we previously observed in brain, liquor and plasma samples. We also used an in-vitro model to observe how exosomes production was affected under conditions of increasing levels of LRP8 receptor, γ- Secretase inhibition, APP overexpression, in response to treatments with recombinant human ApoE 2, 3 and 4, and silencing the LRP8 expression.

MATERIALS AND METHODS

The antibodies used were: anti-LRP8 rabbit polyclonal antibody: GS1 (GeneScript, USA 1:1000), previously developed, targeted to a specific C-terminal protein sequence (**Fig n.2**). GS1 antibody can effectively identify both LRP8 fragments, derived from C-terminal proteolytic processing, and receptor full-length (FL). A mouse monoclonal antibody anti ApoE (WUE-4, Novus Biologicals, 1:1000), a mouse monoclonal anti β-Tubulin antibody (Sigma, T5201 1:2000), a rabbit monoclonal antibody direct against CD9 protein, highly expressed on the exosomes surface (92726Abcam 1:1000), and ALIX (sc-53538 Santa Cruz, 1:500) a mouse monoclonal antibody directed against Alix protein, expressed on the exosomes surface.

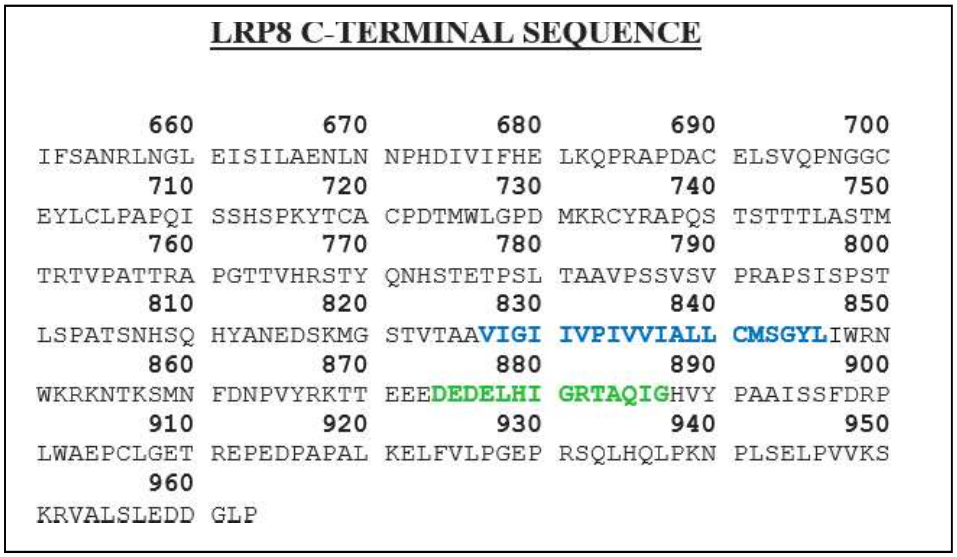

Figure-2 Schematic view of proximal N-terminal, transmembrane and Cterminal regions of the LRP8 isoform 1: blue sequence is the LRP8 transmembrane region, and green region is the sequence used as immunogen to produce our antibody anti-LRP8 (GS1).

Human brain samples

Human cerebral cortex samples were obtained at autopsy from neuropathologically and clinically verified cases sporadic AD (SAD, total n. 6, average age 82.6), familiar AD (FAD, total n. 4, average age 43 y.o.), according to CERAD criteria [96], and aged non-demented subjects (n=6, average age 72.3), in which AD diagnosis has been excluded by clinical evaluation, autopsy examination and immunohistochemical analysis, as described in **Table 1**. Frozen brain samples derived from the Brain Bank of Case Western Reserve University, Cleveland, OH, USA, from the Joseph and Kathleen Bryan Alzheimer's Disease Research Center at Duke University Medical Center, Durham, NC, USA and from the Human Brain and Spinal Fluid Resource Center at University of California, Los Angeles, CA, USA. One piece of grey matter from frontal and temporal cortices were excised under sterile conditions and collected in a single sterile test tube.

Cell cultures and transient transfection

Neuro 2A wild-type cells were plated at a confluence of 1.500.000 in a 10 cm Petri dish.

Neuro 2A wild-type cells were transiently transfected with the plasmid encoding for human APP 695, were grown in DMEM (Dulbecco's Minimum Eagle Medium) supplemented with 10% Fetal Bovine Serum (FBS) decomplemented at 56°C for 30', 1% L-glutamine (2 mM in 0.85% NaCl), 1% penicillin (50 U/L) and streptomycin (50 µg/mL) in a humidified atmosphere at 37°C with 5% CO_2. Neuro 2A wild-type cells were transfected with human APP 695 using Lipofectamine 3000 (Thermo Fisher Scientific, USA), according to the manufacturer's protocol.

Cell cultures and stably transfection

Neuro 2A wild-type cells were plated at a confluence of 1.500.000 in a 10 cm Petri dish.

Neuro 2A wild-type were stably transfected with the plasmid encoding for human LRP8 (Neuro 2A LRP8 DDK-myc and Neuro 2A LRP8 HA), were grown in DMEM (Dulbecco's Minimum Eagle Medium) supplemented with 10% Fetal Bovine Serum (FBS) decomplemented at 56°C for 30', 1% L-glutamine (2 mM in 0.85% NaCl), 1% penicillin (50 U/L) and streptomycin (50 µg/mL) in a humidified atmosphere at 37°C with 5% CO_2. Neuro 2A cells were transfected with hLRP8 DDK myc and hLRP8 HA using Lipofectamine 3000 (Thermo Fisher Scientific, USA), according to the manufacturer's protocol.

Neuro 2A LRP8 DDK myc and Neuro 2A LRP8 HA cells were cultured with a selection of G418 antibiotic, concentrated [0.2] ug/uL.

LRP8 silencing

Neuro 2A wild-type cells were plated at a confluence of 1.500.000 in a 10 cm Petri dish.

Neuro 2A wild-type were transient transfected with the plasmid encoding for human shLRP8 (Origene TR303478), and they were grown in DMEM (Dulbecco's Minimum Eagle Medium) supplemented with 10% Fetal Bovine Serum (FBS) decomplemented at 56°C for 30', 1% L-glutamine (2 mM in 0.85% NaCl), 1% penicillin (50 U/L) and streptomycin (50 µg/mL) in a humidified atmosphere at 37°C with 5% CO_2.

Cells were starved for 48h, and were lysed in RIPA 1X buffer, respective surnatant were collected in a single test tube and stored at -20°C.

ApoE treatment

Neuro 2A wild-type cells and Neuro 2A LRP8 DDK myc were plated at a confluence of 1.500.000 in a 10 cm Petri dish.

Cells were starved and treated for 48h with 20 ug of recombinant human ApoE2, ApoE3 and ApoE4 (PeproTech), respectively. Cells were lysed in RIPA 1X buffer, respective surnatant were collected in a single test tube and stored at -20°C.

DAPT treatment

Neuro 2A wild-type cells were plated at a confluence of 1.500.000 in a 10 cm Petri dish.

Cells were starved and treated for 16h with 10 uM of DAPT. Cells were lysed in RIPA 1X buffer, respective surnatants were collected in a single test tube and stored at -20°C.

Exosomes enrichment from cell culture media

Exosomes were isolated from cell culture media at 4°C, according to protocol described By Thierry et al., [97], collected in a single test tube in PBS 1X, and stored at -80°C. Respective cell cultures were lysed in RIPA 1X buffer, collected in a single test tube and stored at -20°C.

Exosomes enrichment from human brain tissues

Exosomes were isolated from frontal and temporal cortices samples at 4°C, according to protocol described by Vella et al., [98], collected in a single test tube in PBS 1X, and stored at -80°C. Respective frontal and temporal cortices samples were lysed in RIPA 1X buffer, collected in a single test tube and stored at -20°C.

Human brain derived exosomes characterization

Extracellular vesicle concentration and size distribution (size and number of events) were assessed by Nanoparticle Tracking Analysis (NTA) by NS300 instrument (Malvern, Worcestershire, UK) and NanoSight NTA 3.2 Dev Build 3.2.16 software. The system is equipped with a Blue 488 nm laser and a syringe pump system, with a pump speed of 30ml/min. Nanoparticles were illuminated by the laser and their movement under Brownian motion was captured. The samples were measured with manual shutter and gain adjustments: camera level was set at 14 and an analysis detection threshold of 4 used for every sample to provide the relevant comparisons. Background measurements were performed with filtered PBS. All samples were diluted with sterile PBS (1:200) in order to reach a particle concentration suitable for analysis with NTA (10^7–10^9 particles/ml). Diluted samples, prior resuspension, were loaded into the sample chamber with 1ml sterile syringes (BD II, New Jersey, USA). The samples were measured with manual shutter and gain adjustments. All measurements were performed at room temperature. Results were normalized on the weight of each brain sample.

Case	Mental status	Sex	Age	Amyloid plaques	Sample weight (Mg)	ApoE	Notes
4294	Normal	M	84	Negative	312	3/3	
4308	Normal	M	70	Negative	212	33	
A94-122	Normal	F	82	Negative	1002	3/3	Aging
A86076	Normal	F	84	Negative	1367	N.A.	Malignant hyperpirexia
984	Normal	F	65	Negative	821	3/3	
09435	Normal	F	49	Negative	243	N.A.	
3244	SAD	F	79	+++	345	3/4	B&B VI
A-979	SAD	N.A.	89	++	1003	N.A.	B&B III
3233	SAD	F	78	+++	206	N.A.	B&B V
A97328	SAD	M	82	N.A.	906	N.A.	N.A.
960	SAD	F	88	++	845	4/4	B&B V
901	SAD	F	80	++	725	4/4	B&B V
124	FAD	M	40	+++	1080	3/3	B&B V
734	FAD	M	41	++	936	2/3	B&B V
313	FAD	F	48	N.A.	715	3/4	B&B IV
967	FAD	F	43	+++	437	3/4	B&B VI

Table 1– Demographic and clinical information about patients analyzed. + to +++ symbols indicate the crescent quantity of amyloid plaques. N.A.= not available.

Cell culture media derived exosomes characterization

Extracellular vesicle concentration and size distribution (size and number of events) were assessed by Nanoparticle Tracking Analysis (NTA) by NS300 instrument (Malvern, Worcestershire, UK) and NanoSight NTA 3.2 Dev Build 3.2.16 software. The system is equipped with a Blue 488 nm laser and a syringe pump system, with a pump speed of 30ml/min. Nanoparticles were illuminated by the laser and their movement under Brownian motion was captured. The samples were measured with manual shutter and gain adjustments: camera level was set at 14 and an analysis detection threshold of 4 used for every sample to provide the relevant comparisons. Background measurements were performed with filtered PBS. All samples were diluted with sterile PBS (1:100) in order to reach a particle concentration suitable for analysis with NTA (10^7–10^9 particles/ml). Diluted samples, prior resuspension, were loaded into the sample chamber with 1ml sterile syringes (BD II, New Jersey, USA). The samples were measured with manual shutter and gain adjustments. All measurements were performed at room temperature. Results were normalized on the weight of each brain sample. Results were normalized on the concentration of the relative protein lysate.

SDS-PAGE and Western Blots experiments

Western Blot (WB) experiments were performed using human brain derived exosomes, and Neuro 2A wild-type, LRP8-HA and DDK myc derived exosomes.

Samples protein amount was determined using Bradford (Bio-Rad, Italy) and them were loaded with Sample Buffer 2x (SDS 8%, glycerol 24%, Tris 100 mM, tricine 100 mM, dithiothreitol 15 mg, Coomassie brilliant blue g-250 0.05%) in a Tris-Tricine SDS PolyAcrylamide Gel Electrophoresis (SDS–PAGE), with different % of acrylamide according to the experimental needs, and transferred to a PVDF membrane 0.22 μm (Amersham, UK). Membranes were blocked by an incubation of 2 h in Phosphate-buffered saline Tween-20 (PBS-T) containing 5% non-fat dried milk and blotted over night with the primary antibodies previously described.

After washing with PBS-T, the membranes were incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washing the reactive bands were revealed with ECL Plus Western Blotting Detection Reagents (Amersham, UK).

Densitometric analysis of protein bands was performed using ImageLab software system (Bio-Rad, Italy).

Statistical analysis

Tukey's multiple comparisons test with the threshold set at $p<0.05$ (*=$p<0.05$;**=$p<0.01$;***=$p<0.001$,****=$p<0.0001$), C.I. 95%, to evaluate the statistical significance in the analysis of more than two different groups. All the analyses were performed using GraphPad Prism (GraphPad Software, version 8 USA).

RESULTS

Exosomes production dramatically decreases in FAD and SAD brain vs non AD brain

Starting from the ex-vivo tissue analysis, we decided to isolate brain exosomes derived from patients with SAD and FAD and from non-demented subjects, to observe if there were variations in the amount of exosomes produced in case of disease.

We analyzed exosomes derived from 5 control subjects, 6 patients affected by SAD and from 4 FAD patients.

The exosomes isolated from human brain samples (see materials and methods), were then analyzed by Nanosight. To analyze the relative production of exosomes from each starting sample, we normalized the data from analysis with Nanosight to the weight of each brain sample.

We observed that exosomes production significantly decrease in human brain derived exosomes (hBDEs) from SAD and FAD compared to controls.

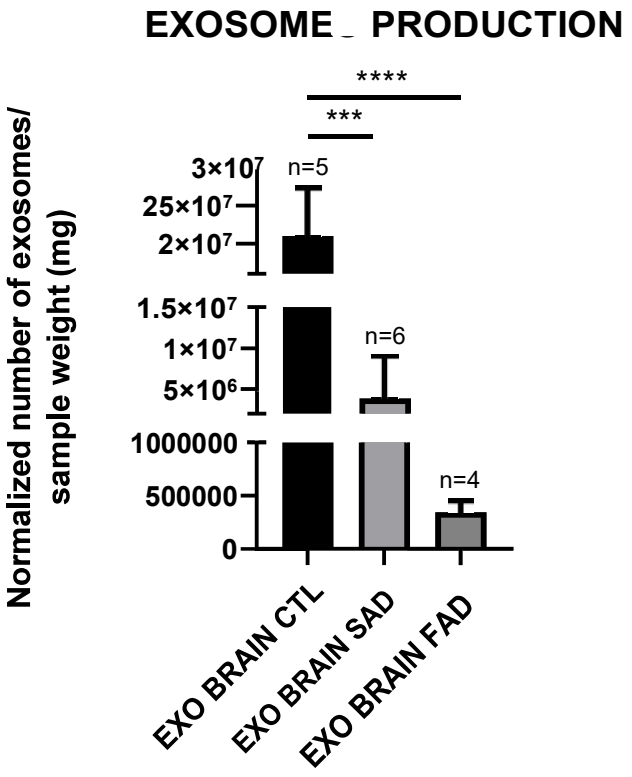

Figure 3- Analysis of human Brain Derived exosomes with NanoSight. The number of exosomes isolated from brain samples derived from patients affected by SAD is significantly lower than the number of exosomes isolated from brain samples from non-demented subjects. This reduction is even more significant if we analyze the amount of exosomes isolated from brain samples derived from patients affected by FAD. Data were normalized on the weight of each brain sample, from which the exosomes were isolated.

Exosomes isolated from SAD and FAD patients contain large amounts of LICDs

After observing a strong reduction in the number of hBDEs isolated from patients affected by SAD and FAD, we conducted western blot experiments on the same exosomes, in order to study their content, in terms of LRP8 receptor.

We observed that, in exosomes derived from SAD patients, LICDs are present in an evident manner, with a molecular weight ranging from 12 to 15 kDa, detected by GS1 antibody.

These LICDs have never been described in the literature, but our previous studies had also highlighted them in cerebral lysates of patients affected by both SAD and FAD.

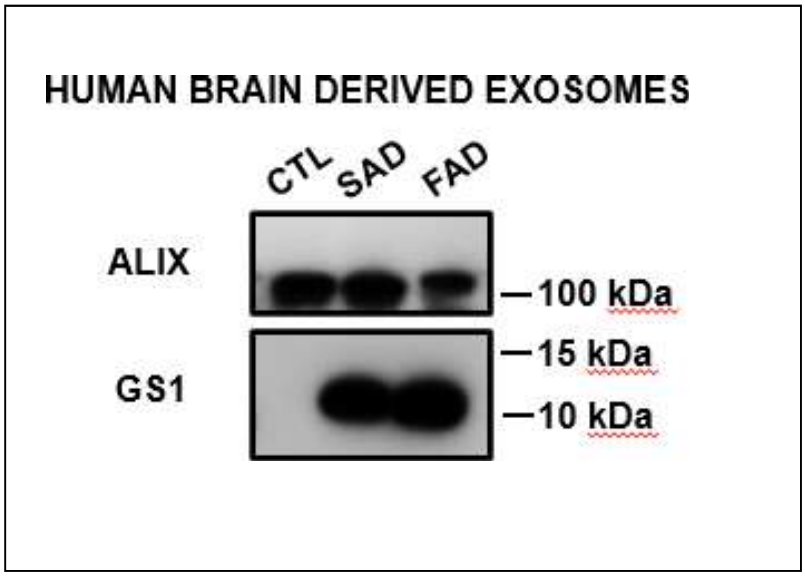

Figure 4- Representative WB of hBDEs. 1 case of control non-demented, 1 SAD case and 1 FAD case revealed a significant presence of LICDs in exosomes derived from SAD and FAD patients compared to non-demented control subjects. Densitometric analysis of LICDs levels were normalized on ALIX.

LRP8 expression significantly increases exosome production in Neuro 2A cells

Considering the reduction in the hBDEs derived from SAD and FAD patients, and the altered processing of the LRP8 receptor that we previously observed in hBDEs derived both from SAD and FAD patients, we decided to investigate if the expression of LRP8 was involved in the production of extracellular vesicles, using an in-vitro cellular model. To this poupose we have set up cell cultures of Neuro 2A wild-type cells, stably transfected with LRP8 myc DDK plasmid and stably transfected with LRP8 HA plasmids.

Both of these plasmids express the same hLRP8 sequence, but they have different tag, in different position; DDK myc tag is in C-terminal region, instead the HA (Human influenza hemagglutinin) tag is in N-terminal region. We observed that the production of exosomes increases significantly expressing the LRP8 receptor, both with DDK myc and HA tag.

Data were normalized quantifying total number of exosomes, on the total proteins present in the corresponding cell lysate.

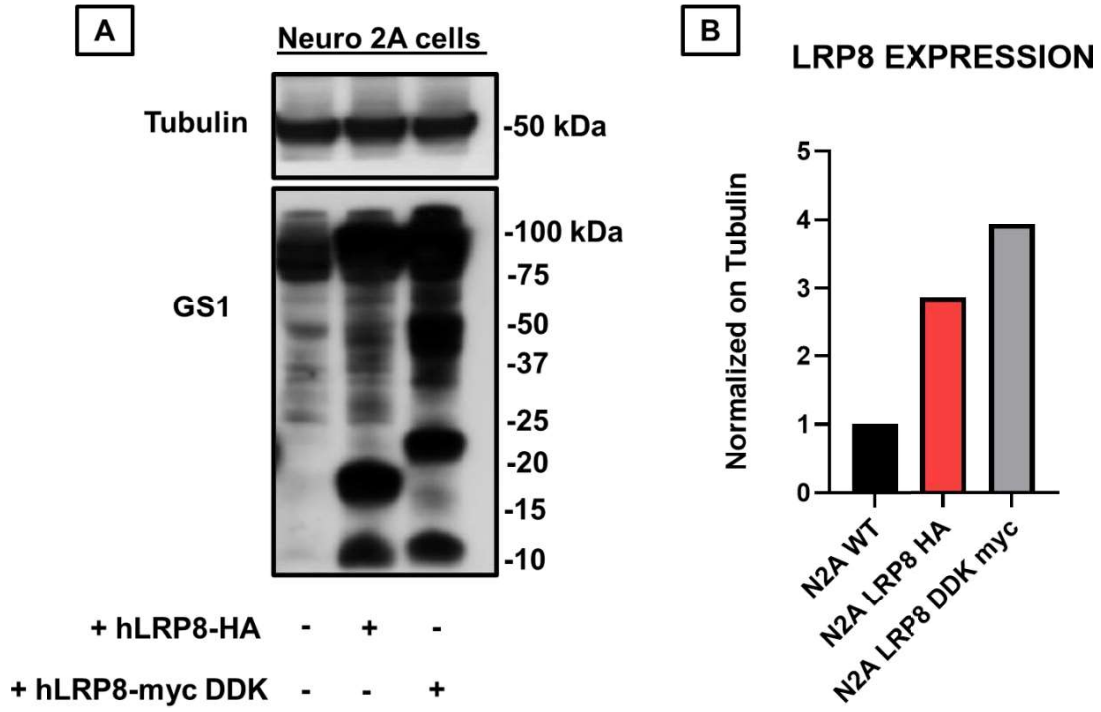

Figure 5- (A) A Representative WB of Neuro 2A wild-type, Neuro 2A LRP8 HA and Neuro 2A LRP8 DDK myc. (B) LRP8 total expression in Neuro 2A wild-type (N2A WT), Neuro 2A LRP8 HA and Neuro 2A LRP8 DDK myc, obtained by Densitometric analysis of LRP8 protein levels normalized on β-tubulin.

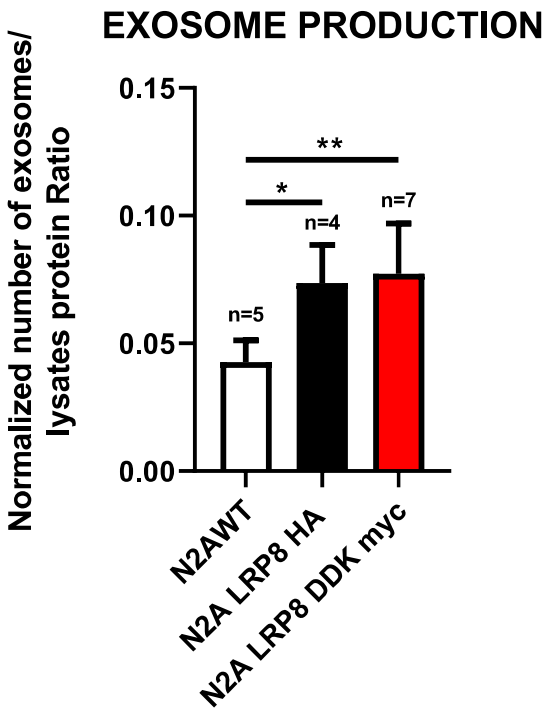

Figure 6 - Analysis of exosomes derived from Neuro 2A wild-type, Neuro 2A LRP8 HA and Neuro 2A LRP8 DDK myc. Exosome production is significantly increased by LRP8 expression both with HA and myc-DDK tag, compared to endogenous expression of LRP8 in Neuro 2A wild-type. Data were normalized on respective lysates protein.

DAPT treatment drammatically decrease exosomes production in-vitro

Previous studies have shown that the C-terminal fragments of LRP8 migrate between 25 and 20 kDa and from them derive fragments between 15 kDa and 19 kDa, through the action of γ-Secretase complex, as reported in the literature [99; 107].

On the contrary, in our previous studies, we have observed in ex-vivo tissues and in cell models, LICDs that migrate between 8-12 kDa, even under conditions of γ-Secretase block, using pharmacological inhibitors. The hypothesis is, therefore, that these fragments do not derive from the action of γ-Secretase and the inhibition of γ-Secretase would promote the action of other enzymes able to process LRP8, generating, at the same time, the accumulation of their precursors. We therefore decided to treat N2A wild-type, Neuro 2A LRP8 HA and Neuro 2A LRP8 DDK myc cells, with DAPT to analyze the effect of γ-Secretase blockade, on exosomes production, trying to imitate a possible condition of loss of functions of γ-Secretase.

Analyzing by WB both cells lysates and relative isolated exosomes, we observed that, in the presence of DAPT, in cell lysates an accumulation of fragments between 20 and 25 kDa (γ-Secretase substrates), is evident, especially in Neuro 2A LRP8 DDK myc. Fragments with a molecular weight of less than 15 kDa are present, also in the respective exosomes. The effect of the DAPT, moreover, significantly reduce the production of exosomes in all three cell lines used.

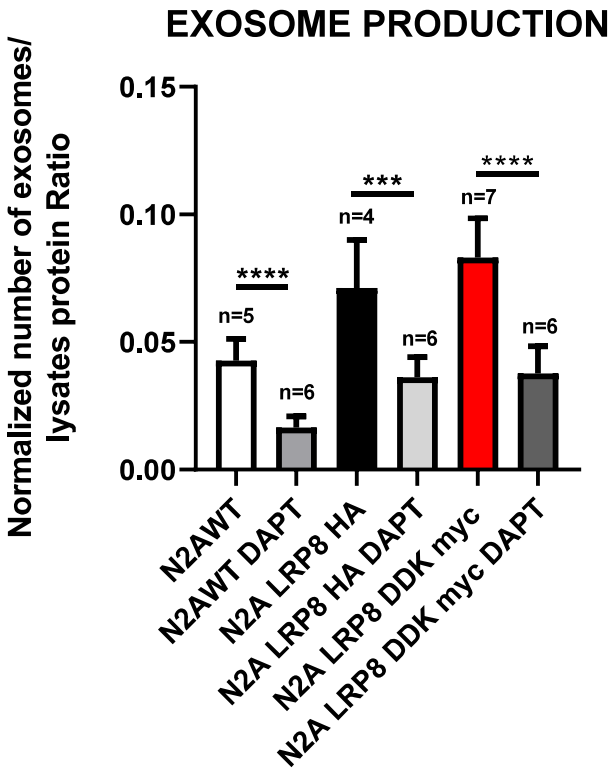

Figure 7- Analysis of exosomes derived from Neuro 2A wild-type, Neuro 2A LRP8 HA and Neuro 2A LRP8 DDK myc treated with DAPT. Exosomes production is significantly decreased by DAPT treatment in Neuro 2A wild-type, Neuro 2A LRP8-HA and Neuro 2A LRP8 DDK myc. Data were normalized on respective lysates protein.

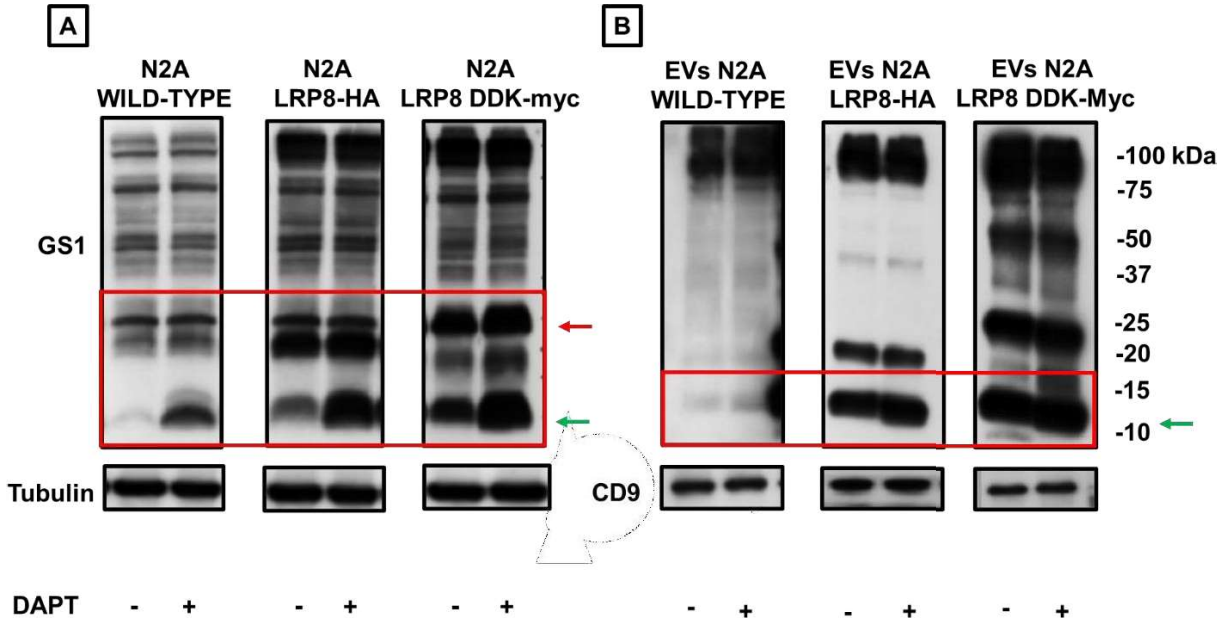

Figure 8- A Representative WB of Neuro 2A wild-type, Neuro 2A LRP8 HA and Neuro 2A LRP8 DDK myc. Differences are evidenced among the groups in the LRP8 LICDs, in particular when cells were treated with DAPT. The red arrow indicates the γ-Secretase substrates, the green arrow indicates the LICDs that do not come from the γ-Secretase processing and that increase in all three cell lines. Densitometric analysis of LRP8 LICDs were normalized on β-tubulin. (B) LRP8 LICDs are also evidenced in exosomes derived from the relative cell culture. Densitometric analysis of LRP8 LICDs were normalized on CD9.

APP over-expressions modifies the production of exosomes in Neuro 2A cells and the processing of the LRP8 receptor

Trying to understand in conditions of APP over-expression (such as in DOWN syndrome) and in loss of function of γ-Secretase, how the production of exosomes could undergo changes, and how the processing of the LRP8 receptor could change, we performed experiments using Neuro 2A wild-type cells. We analyzed by WB, cells lysates and exosomes derived from Neuro 2A wild-type transient transfected with APP h695 and treated with DAPT. We observed that the over-expression of APP increase the production of exosomes, and that the simultaneous treatment with DAPT decrease their production. Looking at the processing of LRP8, on the other hand, we observed that in the case of exosomes derived from cells transfected with hAPP 695 and treated with DAPT, the LICDs are more evident, compared to the corresponding cell lysate. The same situation was observed for the C99 fragment resulting from APP processing, which is, therefore, much more evident in the exosomes.

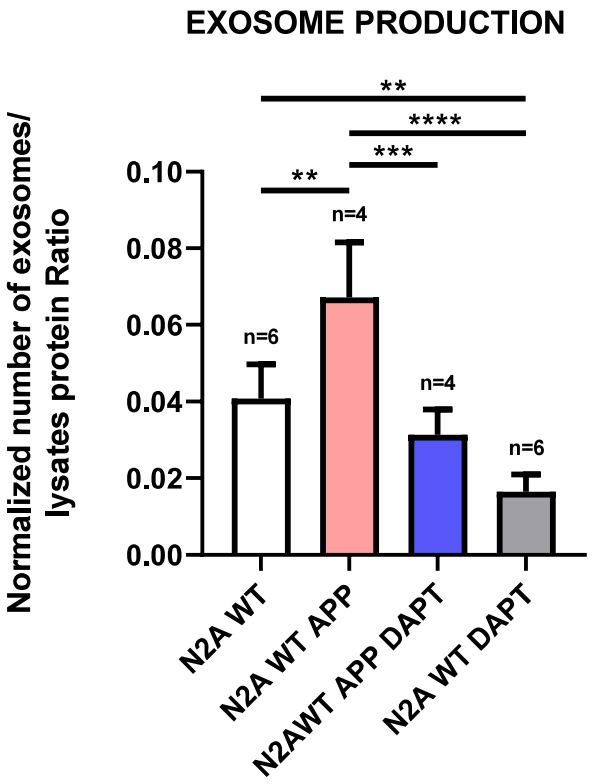

Figure 9- Analysis of exosomes derived from Neuro 2A wild-type, transfected with hAPP and treated with DAPT. Human APP 695 expression significantly increase exosomes production, instead hAPP 695 + DAPT treatment, and DAPT treatment, decrease exosomes production in Neuro 2A wild-type cells. Data were normalized on the relative lysates protein.

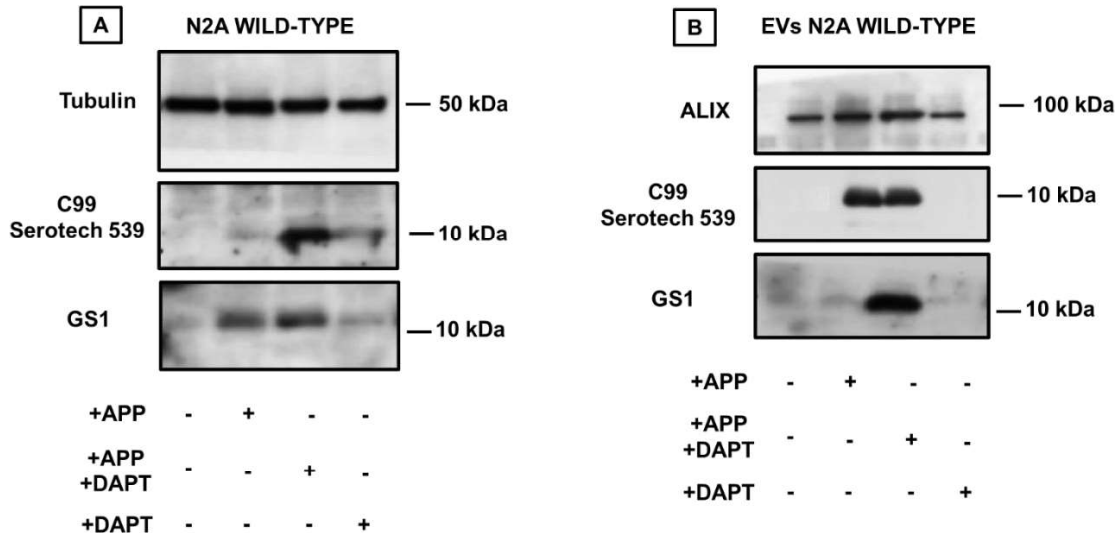

Figure 10 - A Representative WB of Neuro 2A wild-type, transfected with hAPP 695 and treated with DAPT. (A) Significant differences are evident among the groups in the LRP8 LICDs, in particular in cells transfected with hAPP 695 and treated with DAPT. Densitometric analysis of LICDs were normalized on β-tubulin. (B) Significant increase in LICDs are evident in exosomes derived from the relative cell culture. Densitometric analysis reveals a significant increase of LICDs in exosomes derived from cell culture transfected with hAPP 695 and treated with DAPT. Densitometric analysis of LICDs were normalized on ALIX.

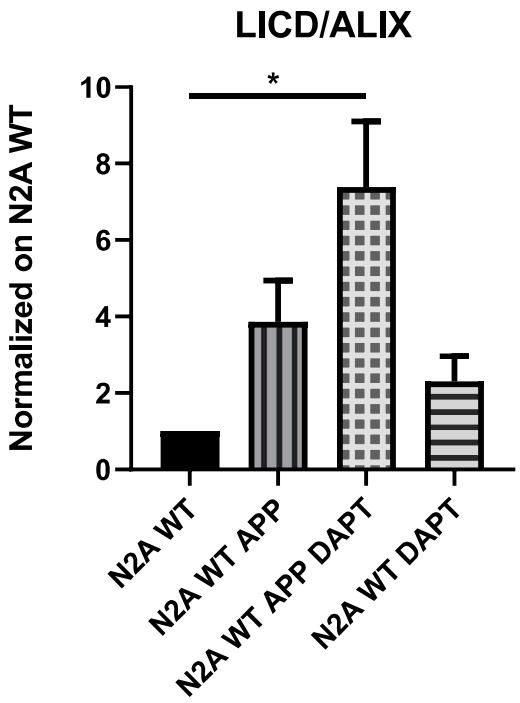

Figure 11- Densitometric analysis of LICDs levels in exosomes derived from Neuro 2A wild-type, transfected with hAPP695 and treated with DAPT. Significant differences are evident among the groups in the LICDs levels. Densitometric analysis reveals a significant increase of LICDs levels, in exosomes derived from Neuro 2A wild-type transfected with hAPP 695 and treated with DAPT together, compared to exosomes derived from Neuro 2A wild-type, transfected with hAPP 695 and treated with DAPT.

ApoE4 significantly promote LRP8 processing in Neuro 2A wild-type derived exosomes and compromize exosomes production

In order to verify a correlation between ApoE, exosomes production and LRP8 processing, we analyzed by WB, cell lysates and exosomes derived from Neuro 2A wild-type, previously treated with recombinant human ApoE2, ApoE3 and ApoE4. Data show that, in presence of the same amount of endocytosed ApoE, the processing of LRP8 receptor in cell lysates, does not change significantly. On the contrary, at the extracellular level, ApoE4 appears to be more present in exosomes. Data were normalized versus CD9. Furthermore, the data show that the E4 isoform promote the processing of LRP8, evident in exosomes, with LICDs between 15 and 10 kDa.

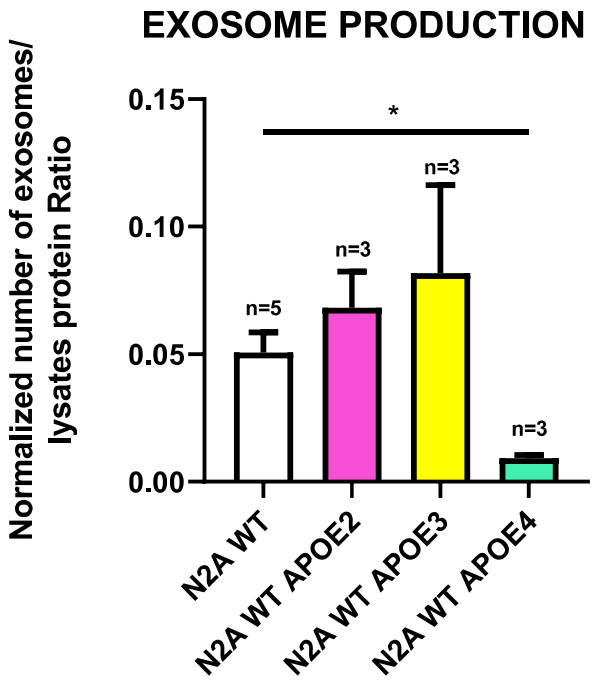

Figure 12 – Analysis of exosomes derived from Neuro 2A treated with recombinant human ApoE2, ApoE3 and ApoE4, with NanoSight. ApoE4 treatment significantly decrease exosomes production in Neuro 2A wild-type cells versus Neuro 2A wild-type non treated. Data were normalized on the relative lysates protein.

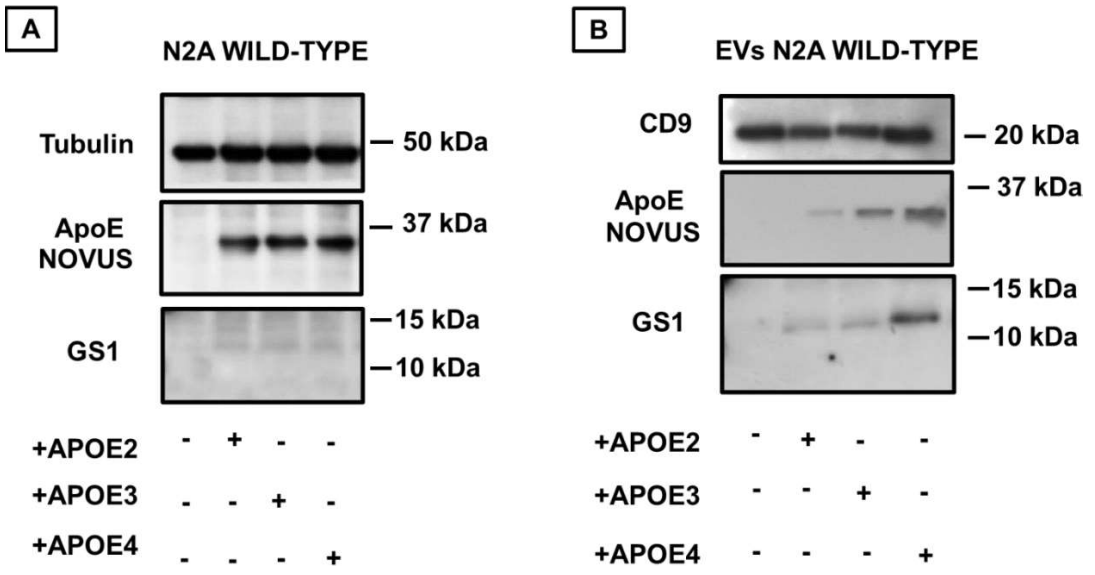

Figure 13 - A Representative WB of Neuro 2A wild-type, treated with recombinant human ApoE2, ApoE3 and ApoE4. (A) No significant differences are evident among the groups in the LICDs, when Neuro 2A wild-type were treated with recombinant human ApoE2, ApoE3 and ApoE4. Densitometric analysis of LICDs were normalized on β-tubulin. (B) Significant increase in LICDs is evident in exosomes derived from cell cultures treated with ApoE4. Significant increase in ApoE level is also evident in exosomes derived from cell cultures treated with ApoE4, compared to ApoE2 and ApoE3 levels. Densitometric analysis of LICDs were normalized on ALIX.

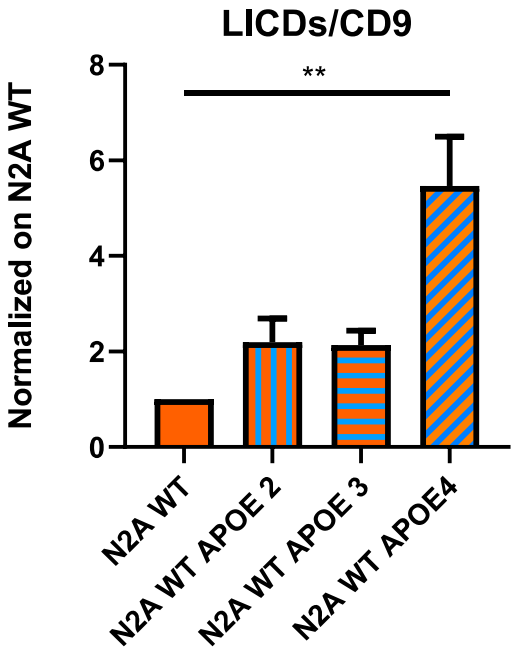

Figure 14 – Densitometric analysis of LICDs levels in exosomes derived from Neuro 2A wild-type treated with recombinant human ApoE2, ApoE3 and ApoE4. Significant differences are evident among the groups in LICDs levels. Densitometric analysis reveals a significant increase of LICDs levels, in exosomes derived from Neuro 2A wild-type treated with ApoE4, compared to exosomes derived from Neuro 2A wild-type treated with ApoE2 or ApoE3.

Apoe4 significantly promote LRP8 processing in Neuro 2A LRP8 DDK myc derived exosomes and compromize exosome production

In order to confirm a correlation between ApoE4 isoform, exosomes production and LRP8 processing, we analyzed by WB, cell lysates and exosomes derived from Neuro 2A DDK myc previously treated with recombinant human ApoE2, ApoE3 and ApoE4. Data show that, in presence of the same amount of endocytosed ApoE, the processing of LRP8 receptor does not change significantly. On the contrary, at the extracellular level, ApoE4 appears to be more present in exosomes. Data were normalized versus CD9.

Furthermore, the data show that the E4 isoform promote the processing of LRP8, evident in exosomes, with LICDs between 12 and 8 kDa.

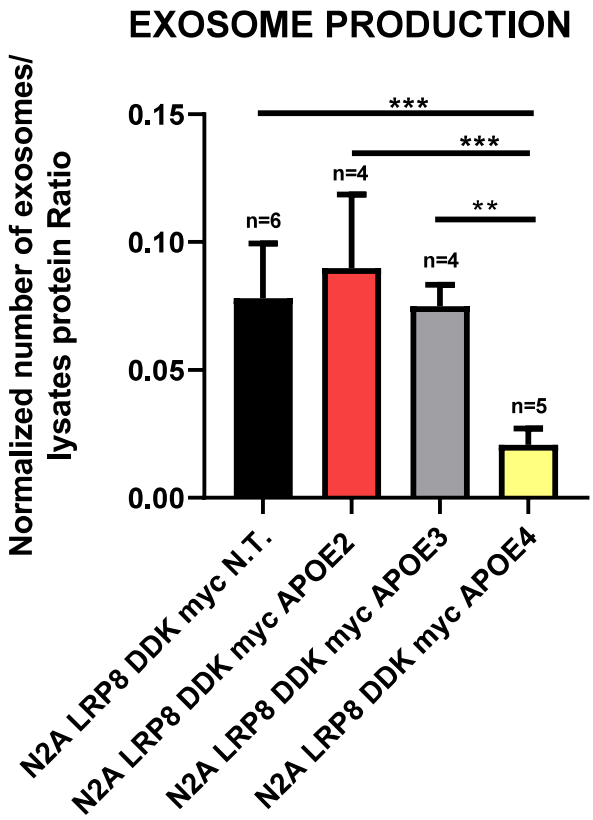

Figure 15 - Analysis of exosomes derived from Neuro 2A DDK myc treated with recombinant human ApoE2, ApoE3 and ApoE4, with NanoSight. ApoE4 peptide treatment significantly decrease exosomes production in Neuro 2A LRP8 DDK myc cells versus Neuro 2A LRP8 DDK myc treated with ApoE2, ApoE3 peptide, and non treated. The data were normalized on the relative lysates protein.

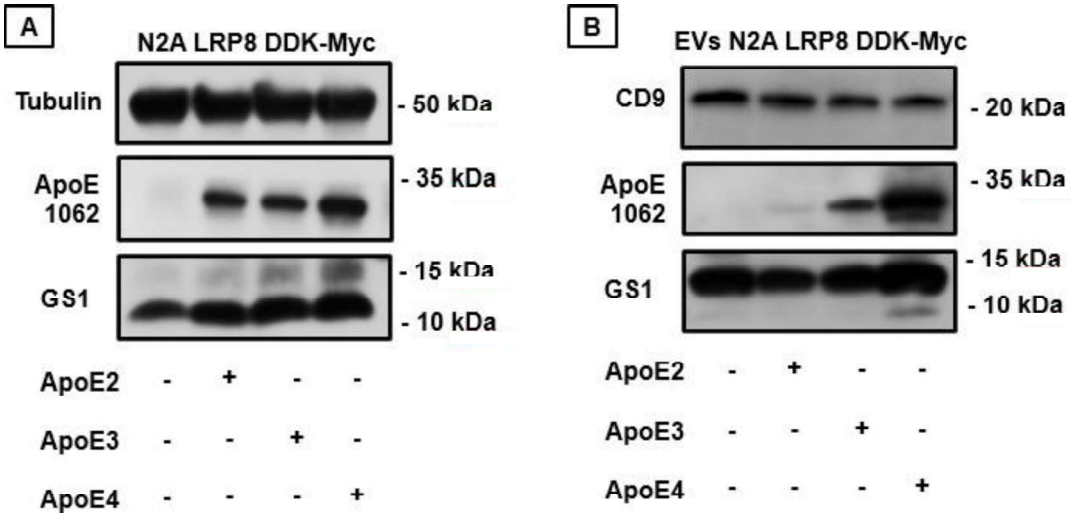

Figure 16 - A Representative WB of Neuro 2A LRP8 DDK myc, treated with recombinant human ApoE2, ApoE3 and ApoE4. (A) No significant differences are evidenced among the groups in LICDs, levels when Neuro 2A LRP8 DDK myc cells were treated with ApoE2, ApoE3 and ApoE4. Densitometric analysis of LICDs were normalized on β-tubulin. (B) Significant increase in LICDs are evident in exosomes derived from cell cultures treated with ApoE4. Significant increase in ApoE level is also evident in exosomes derived from cell cultures treated with ApoE4, compared to ApoE2 and ApoE3 levels. Densitometric analysis of LICDs were normalized on CD9.

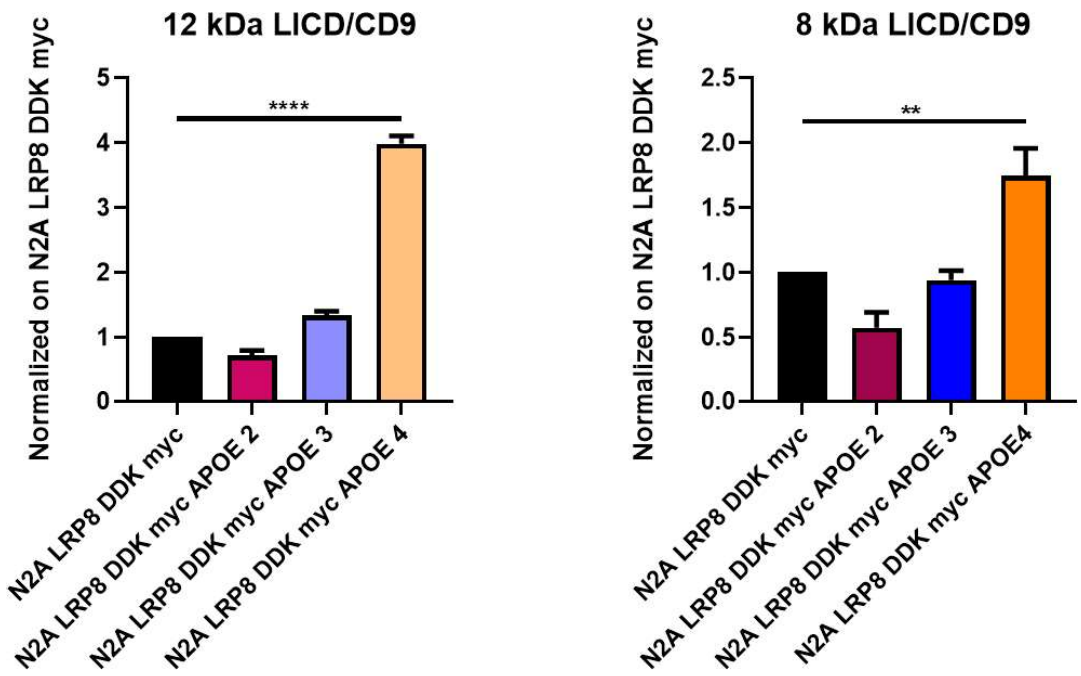

Figure 17 - Densitometric analysis of LICDs levels in exosomes derived from Neuro 2A LRP8 DDK myc treated with recombinant human ApoE2, ApoE3 and ApoE4. (A) Significant differences are evident among the groups in the levels LICDs of 12 kDa. Densitometric analysis reveals a significant increase of LICDs levels, when cell cultures were treated with ApoE4 peptide, compared to exosomes derived from Neuro 2A LRP8 DDK myc treated with ApoE2 and ApoE3. (B) Significant differences are evident among the groups in the levels LICDs of 8 kDa. Densitometric analysis reveals a significant increase of LICDs levels, when cell culture were treated with ApoE4 peptide, compared to exosomes derived from Neuro 2A LRP8 DDK myc treated with ApoE2 and ApoE3.

LRP8 silencing reduce exosome production in Neuro 2A wild-type cells

Based on the observed results, an involvement of the LRP8 receptor in the production of exosomes was evident. In particular, observing how the production of exosomes increased as the expression of LRP8 increases. To demonstrate that LRP8 plays a role in exosomes production, we performed transient silencing on the receptor, using Neuro 2A wild-type, and from the culture media of the transfected cells, we isolated the exosomes, with the protocol previously used. Analyzing by WB the cell lysates of the Neuro 2A wild-type silenced for LRP8 (Neuro 2A sLRP8), we detected a decrease of about 30% of the receptor, and analyzing the exosomes deriving from the respective cell culture media, we found a significant reduction of the number of them, compared to exosomes deriving from Neuro 2A wild-type.

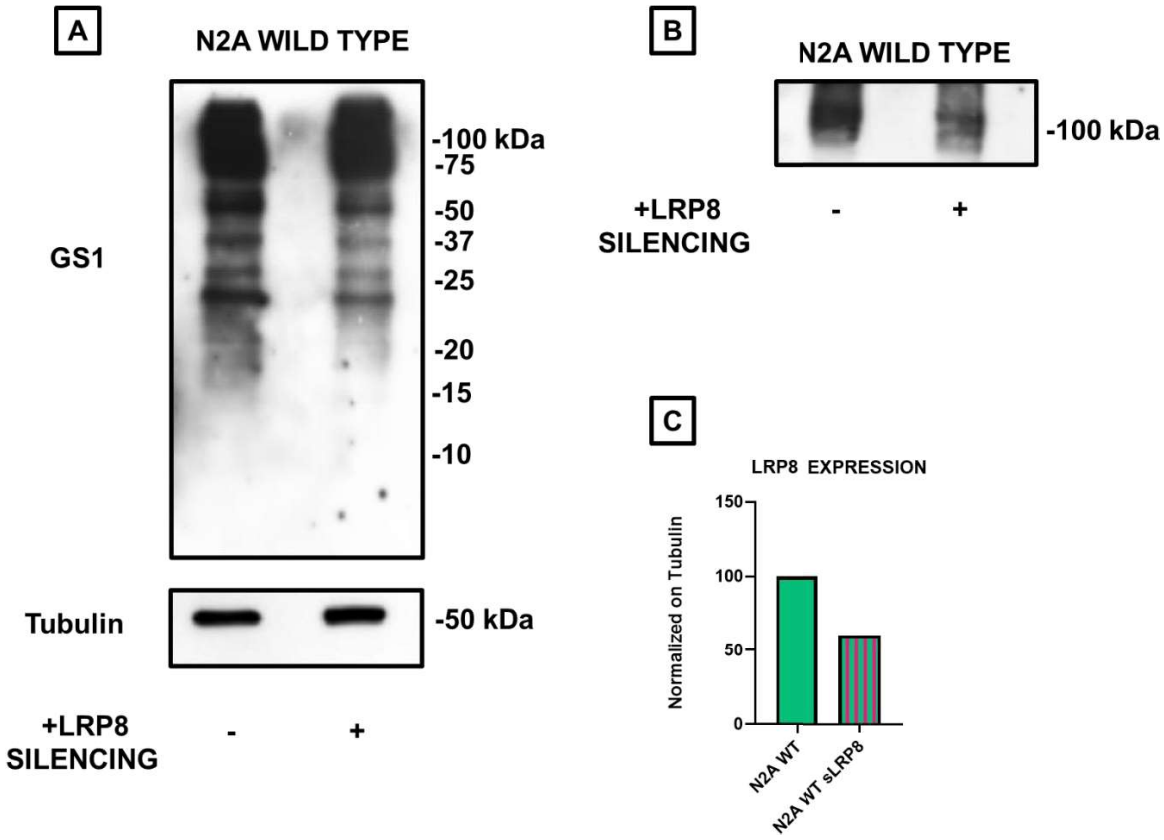

Figure 18 - Representative WB of Neuro 2A wild-type, and Neuro 2A transient transfected with LRP8 silencing and relative quantization. (A) Total amount of the LRP8 receptor, wild-type and with silencing. (B) Particular of the whole form of the LRP8 receptor, at 100 kDa, wild-type and with silencing. (C) Percentage of receptor silenced, compared with the wild-type form of the receptor. The percentage of silencing obtained is 30%, normalizing the values of the densitometric analysis on tubulin.

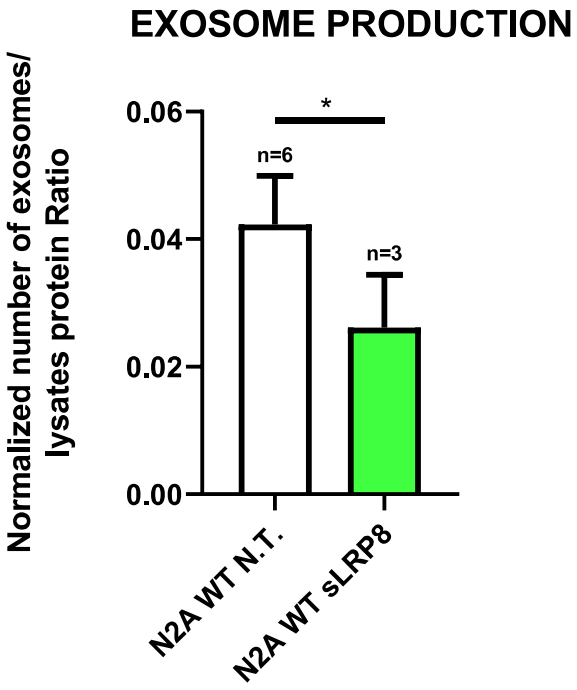

Figure 19 - Analysis of exosomes derived from Neuro 2A wild-type and Neuro 2A wild-type sLRP8 with NanoSight. LRP8 silencing significantly decrease exosomes production in Neuro 2A wild-type cells (sLRP8) versus Neuro 2A wild-type non trasfected (N.T.). Data were normalized on the relative protein lysates.

DISCUSSION

The alterations of the neuronal receptors involved in Alzheimer's disease are well known. Although great efforts have focused on APP, it is also known that therapeutic approaches based on the amyloid theory have proved unsuccessful.

In a complex environment like the human brain, no protein is alone. The brain is a highly complex environment, whose numerous variables change in relation to the external and internal signals. In this highly specialized and variable system, intercellular communication remains a very important aspect. In the last decade, the interest of the scientific community towards exosomal vesicles has enormously grown, in particular in neurodegenerative diseases. In a complex disease such as Alzheimer's dementia, study the role of exosomes, could clarify through which systems the cells communicated with each other, and thinking about the various histopathological manifestations of the disease, what is the consequence of each other, even more ambitiously try to identify the *primum movens* of the disease.

it has been demonstrated that beta-Amyloid peptides accumulate in MVBs besides the capacity to induce apoptosis of astrocytes, thus impairing neuronal functions during the progress of the disease [108; 109].

Different proteases implicated in AD have been found in exosomes: PSEN1 and PSEN2, BACE1, Adam10 [110], are just some examples.

On the other hand, it has been demonstrated that exosomes are able to enhance beta-Amyloid uptake into microglia and dramatically decrease extracellular levels of beta-Amyloid itself [111] even if the exact mechanisms underlying the protective effect remaine unknown.

Exosomes contain a moltitude of different molecules that exert protective actions. Cystatin C, for example, a molecule with several neuroprotective properties, has been shown to be secreted via exosomes [112] or neprilysin, known to be a relevant beta-Amyloid degrading enzyme in the brain [113].

Furthermore, considering that another aspect of the disease is represented by cerebral inflammation, it is believed that exosomes also play a role in the diffusion of toxic species, thus participating in the inflammatory process itself [114].

It is not surprising, therefore, that the interest in these small extracellular vesicles, in the context of Alzheimer's disease, and other neurodegenerative diseases, has grown exponentially in the last 10 years. It has recently been observed that in post-mortem human tissue and humanized mouse models for apolipoprotein E, the expression of apolipoprotein E4 reduced the production of brain exosomes [115]. Considering that the E4 genotype represents a major risk factor for the development of LOAD, it is likely that when brain exosomes production is impaired, the cells lose their efficiency in eliminating materials from the endosomal-lysosomal system.

Disruption of these systems can therefore interfere with neurons ability to degrade material resulting from cellular metabolism.

These events lead to greater neuronal vulnerability and, in the context of Alzheimer's disease, these observations suggest that the dysfunction of the exosomal pathway is an important component of brain pathology and that it is interesting to investigate their role, and which factors intervene in the production of these extracellular vesicles.

In this scenario, it is evident that in a pathological condition, intercellular communication is in some way modified, as well as the endocytic protein

sorting. A feature common to many neurodegenerative diseases is therefore the accumulation of protein aggregates within neurons, in glial cells, and a progressive deposition within distinct brain regions, which ultimately, they are responsible for the clinical phenotype of the disease.

In diseases such as AD, endosomal trafficking disturbance has been observed to be very evident [116] and some observations derive from post-mortem brain tissue analysis of patients, in which aberrant enlargement of early endosomes has been shown [117].

BACE levels have been observed to be elevated in the brains of AD patients. One of the mechanisms underlying this phenomenon is due to the depletion of the GGA3 protein, involved in the transposition of proteins from Golgi to lysosomes [118].

Alterations in protein sorting have also been observed in neurodegenerative diseases such as Parkinson Disease: a point mutation within one of the units forming the retromer, a protein complex involved in the trafficking of proteins within the endosomes, has been linked to the manifestations of Parkinson Disease. It has been demonstrated that its expression interrupts the traffic of cathepsin D, a protease responsible for the degradation of α-synuclein and overexpressed in Alzheimer's disease [119; 120].

A greater understanding of the proteins involved in endocytic trafficking, and of the regulation of proteins potentially linked to neurodegenerative diseases would provide new clinical and diagnostic tools.

In this thesis, we decided to investigate whether the neuronal receptor LRP8 was involved in the production of exosomes, from ex-vivo tissues and using an in-vitro cell model.

From the observations of experiments conducted on exosomes isolated from human brain tissues we observed that in the case of SAD, the production of exosomes decreases significantly compared to controls, and that this reduction is even more significant when we analyze the number of exosomes isolated from brain deriving from FAD patients. Not only a significant variation in the number of extracellular vesicles produced was highlighted in the case of AD, but also the contents of the vesicles themselves, is strongly different in case of disease, compared to the samples deriving from non-demented subjects.

The presence of LICDs highlighted both in hBDEs derived from SAD patients, and in hBDEs derived from FAD patients but not in hBDEs derived from non demented subject, highlights how the receptor processing is altered in the case of AD.

Considering this remarkable difference in the content of isolated extracellular vesicles, regarding LRP8 receptor, it is plausible to believe that in the case of dementia, the C-terminal fragments, which accumulate in the exosomes, could have a pathological significance acting as a signal of disease on surrounding cells.

This hypothesis is supported, in a certain way, by the results obtained in-vitro. The effect of the ApolipoproteinE4 on the processing of LRP8 is very evident in exosomes derived from Neuro 2A wild-type and Neuro 2A LRP8 DDK myc, highlighted by the presence of 12 and 8 kDa LICDs.

In this case, perhaps it would be more correct to speak of a different sorting of LICDs, since in the corresponding cell lysate no significant differences in the proteolytic processing of the LRP8 receptor were highlighted, contrary to what was observed in the related exosomes.

We know that the expression of the E4 isoform of the Apolipoprotein E is linked to an increased risk of developing Alzheimer's dementia, therefore, starting from this assumption, it is plausible think that the role of C-terminal fragments deriving from the proteolytic processing of LRP8, could have a pathological significance in the disease, although in our study we did not find pathological effects of LICDs, for example on tau or on beta-Amyloid peptides.

Analyzing the number of exosomes produced by the cell cultures used, we found a significant decrease in isolated exosomes, following treatment with the recombinant ApoE4, both in the case of exosomes deriving from Neuro 2A wild-type cells and in exosomes derived from Neuro 2A LRP8 DDK myc. The reducing effect on ApoE4 exosome production, however, is supported by the literature. A study showed that in human post-mortem tissue and in models humanized for apolipoprotein E, the expression of APOE4 reduced levels of exosomes in the brain [3] in an age-dependent manner in mice: it was evident at 12 months but not at 6 months of age. Regarding the effect of the ApoE4 allele on the production of exosomes, we know that the expression of ApoE4 is able to cause dysfunctions of the lysosomal endosomal neuronal pathway in mouse models [121; 122]; in the pyramidal neurons of the cingulate cortex of mice expressing the APOE4 allele, the first endosomal changes are evident at 18 months of age.

Hence, a reduction in brain exosome levels precedes neuronal endosomal changes in the brains of APOE4-expressing mice.

Both Apolipoprotein E and LRP8 are introduced into the cell within the endosomal vesicles.

Usually the LRP8 receptor is rapidly recycled to the cell surface, but this recycling process has been observed to occur more slowly in individuals expressing the E4 allele of ApoE [123].

It has been hypothesized that ApoE4 causes the disruption of the normal transport of endosomal vesicles and the recycling of the LRP8 receptor, most likely due to its propensity to unfold and assume a "fused blood cell" conformation upon entering the acidic environment of the early endosome [124]. ApoE4 differs from ApoE3 by a single amino acid, which alters its isoelectric point in so that it coincides with the pH of ~ 6.5, i.e. the pH present in the early endosome [125;126].

By linking our results obtained in vitro, to what is reported in the literature, it is plausible to believe that ApoE4, binding to the LRP8 receptor, with greater affinity than the isoforms ApoE2 and ApoE3 [127] and hindering the recycling of LRP8, remains longer at internal of the endosomal vesicles, undergoes more proteolytic processing, which results in an increase in LICDs, of variable molecular weight, between 12 and 8 kDa, and which accumulate in the exosomes, as we have observed in this thesis work.

Also the experiments conducted on Neuro 2A wild-type transfected with hAPP 695 gave results that support the hypothesis of the probable negative function of LICDs, considering in particular the results obtained in combination with the γ-Secretase inhibitor, DAPT.

Observing the exosomes deriving from Neuro 2A wild-type transfected with hAPP 695 and treated with DAPT, we highlighted a significant presence LICDs, around 12 kDa.

This remember a situation in which γ-Secretase shows a loss of function, similar to what happens in cases of genetic AD, due to mutations in presenilin. In this case, LICDs, also give other informations, considering that they do not derive from the proteolytic processing of γ-Secretase, because it is blocked by DAPT; so, probably, other enzymes are involved in the proteolytic processing of LRP8.

Observing the results of these experiments, we also highlighted that in cell lysates the presence of LICDs is lower than the quantity present in the respective exosomes, in particular following APP overexpression and treatment with DAPT, a sign that these LICDs undergo a different ordering from that observed in the corresponding cell lysate, similar to that observed following treatments with ApoE4.

Regarding the overexpression of APP, and the increase in the production of exosomes, it has been observed that individuals with Down syndrome show an increased exosomes production [128] and that they are larger than the standards [129]. In Down syndrome, endosomal dysfunction is not age-dependent, as observed for ApoE4, but begins before birth [128], causing a greater release of exosomes over the years.

Our results concerning the over-expression of APP and the treatment with ApoE4 go in two different directions, but underline, in accordance with the data in the literature, the importance of endosomal-exosomal regulation.

In addition to the number of exosomes produced in the unit of time, it would also be important to observe the composition of the exosomes, their size and above all their content. In the future, it would be very interesting to study the possible impact that LICDs could have on the aggregation and phosphorylation states of tau, using an animal model.

Given the results obtained so far from our studies, however, we believe that the effect of LICDs present in exosomes should certainly be analyzed in the future, as it could have clinical or even diagnostic value.

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