University of Genoa

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Curriculum: Cellular and molecular pathology in age-related diseases

XXXII Cycle

"cGMP favors the interaction between APP and BACE1 by inhibiting Rab5 GTPase activity"

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Chapter 1

Introduction

1.1. Alzheimer's disease

The first case of Alzheimer's disease (AD) was reported in 1906 by the German neuropathologist Alois Alzheimer (1864-1915) during the Meeting of South-West German Psychiatrists in Tubingen. He described the case of his 50-year-old patient who presented a specific clinical picture: she was suffering from paranoia, progressive sleep, memory disturbance, aggression, and confusion.

After the patient's death, Alzheimer and Gaetano Perusini, an Italian neuropsychiatrist, observed that the psychiatric symptoms could be correlated with the presence of particular cerebral lesions: peculiar plaques and neurofibrillary tangles [1].

Today AD is considered as the most common cause of dementia and it is recognized by the Word Health Organization as a global public health priority.

AD affects about 5% of the population aged over 65 and 40% of the population aged over 80 [2]. More than forty-five million people are affected by the disease worldwide and this number is expected to grow to 131.5 million by 2050.

The costs of coping with AD are enormous and, in 2010 only, 604 billion dollars have been spent. Despite the great force to resist the disease, no therapies have so far been able to prevent AD onset or progression [3-5].

The age, a positive family history and, to a lesser extent, the female sex, are the most significant risk factors, but the exact cause of AD remains unknown.

1.1.1. What is Alzheimer’s disease?

Alzheimer's disease is a progressive, unremitting neurodegenerative disease characterized by cognitive impairment and memory loss. AD is thought to begin 20 years or more before symptoms arise and it has an average clinical duration of 8–10 years.

Generally, the course of the disease is divided into three phases: an initial stage in which there is a slight cognitive deficit, an intermediate stage, until reaching a pathological severity defined as serious in the more advanced stages.

Probably, the essential symptom of the disease, especially in the early stages of illness, is the memory impairment; in fact, although with different gravity, all mnemonic compartments are affected. Initially, there is in fact the tendency to forget apparently trivial everyday life circumstances; this constitutes the basis for those that will be much more severe cognitive deficits, such as, for example, not remembering familiar names or places.

When the disease progresses, the patient shows difficulty speaking (aphasia) and becomes apathetic, feeling no interest for his/her daily activities. Activities that were important to the individual’s identity, such as planning family events or participating in sports, may no longer be possible.

In the intermediate phase of the disease there is a progressive aggravation of the disorder; the subject is unable to learn new information and loses orientation even in familiar environments, due to the space-time disorientation and the early deficits of episodic memory.

In the final stages of AD, people are bed-bound and need round-the-clock care.

Symptoms occur because neurons in regions of the brain involved in thinking, learning and memory have been damaged or destroyed.

In the most serious cases the terminal phase may arrive only after three years from the beginning of disease [4, 6].

1.1.2. Genetics

From the clinical point of view, two different forms of AD pathology are distinguished: the sporadic form (SAD) and the familial form (FAD). SAD, in which symptoms appear at age 65 or older (late-onset), is the most common and includes more than 99% of all cases [7]. The etiology of this form is still unknown and the pathogenesis has yet to be defined. FAD is characterized by autosomal dominant transmission and early onset, in fact individuals tend to develop symptoms before age 65. They are a small percentage of Alzheimer's cases (1% or less) [8] and progress AD as a result of mutations to any of three specific genes.

The first genetic mutation linked to dementia was identified on chromosome 21, in the locus coding for the amyloid precursor protein (APP) [9]. Other mutations have been identified in the genes coding for presenilin 1 (PS1) and presenilin 2 (PS2), two enzymes involved in the production of β-amyloid (Aβ) [4].

Mutations of these three genes are considered the most common cause of early AD, supporting the long-lasting "amyloid cascade hypothesis", which considers Aβ accumulation as the primary cause of AD [10-12].

1.1.3. Morphological alterations

AD is characterized by macroscopic and microscopic alterations.

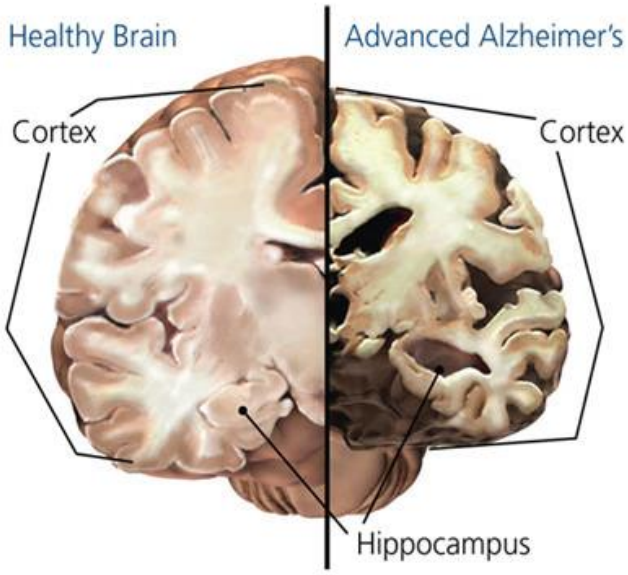

Figure 1 *– Macroscopic morphological alterations of a brain with Alzheimer's disease*

The most evident macroscopic characteristic of the brain of a subject suffering from AD is the marked cortical atrophy, which determines an increased amplitude of the cerebral sulci and the increase of the ventricular volume, as illustrated in **Figure 1**.

This atrophy appears widespread and is mainly linked to neuronal degeneration, which involves the reduction of dendritic spines and synaptic junctions, leading to death of the nerve cell through apoptotic processes.

Studies conducted by Takashi Ohnishi and collaborators on AD brains described an important reduction of grey matter volume in the bilateral hippocampal formation, entorhinal cortex, and parahippocampal cortex [7].

Also Schott and colleagues showed that the mainly affected structures of AD brains are the hippocampus and the para-hippocampal gyrus of the temporal lobe and the cortical associative areas. On the contrary, the posterior areas of the hemispheres, the cerebellum and the brainstem are relatively spared [13].

In 1906 Alois Alzheimer first described the histopathological changes that characterize the disease: neuritic plaques and neurofibrillary tangles **(Figure 2)**.

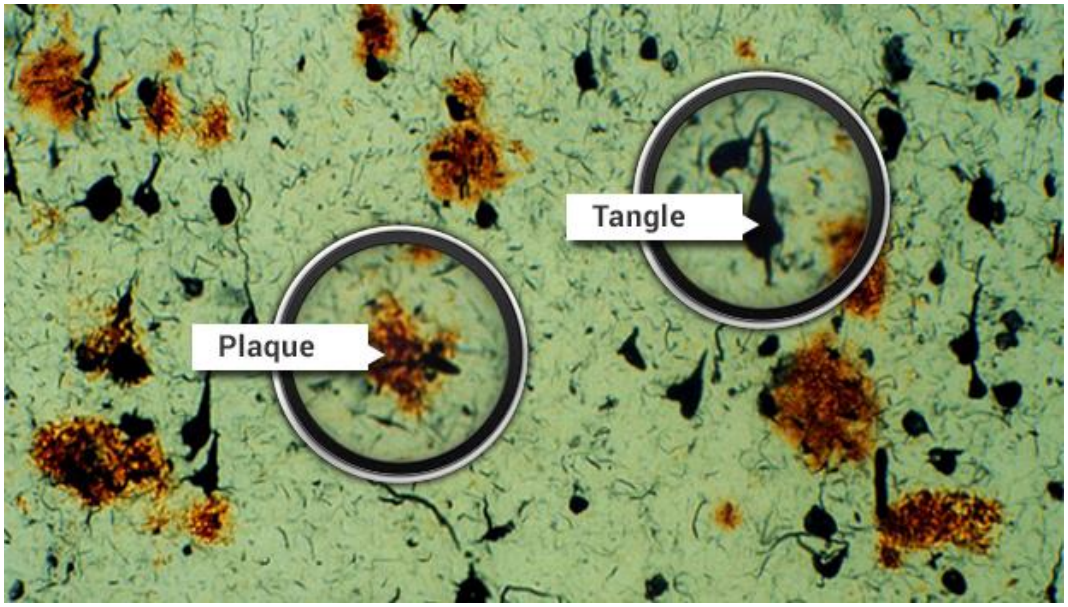

Figure 2 *- Neuritic plaques and neurofibrillary tangles. Immunohistochemistry of a hippocampal section. The anti-β-amyloid antibody reveals neuritic plaques, while the anti-PHF-1 antibody recognizes neurofibrillary tangles.*

Neuritic plaques, also called senile plaques or amyloid plaques [6], are extracellular and roundish structures with a diameter of 50-200 μm, which develop more abundantly in some areas of the brain such as hippocampus, parahippocampus and amygdala. The plaques are mainly constituted of aggregates of a 40-42 amino acids peptide called β-amyloid (Aβ). Around the plaques, two types of activated glial cells are frequently present: microglial cells and astrocytes.

The neurofibrillary tangles (NFT) are intraneuronal aggregates of hyperphosphorylated and misfolded tau, a microtubule-associated protein. NFT accumulate in the cellular body of neurons, mainly in the hippocampus, entorhinal cortex, amygdala and basal forebrain nuclei, but when tangle-bearing neurons die, they become extracellular [14, 15].

1.1.4. How β-amyloid is produced

Aβ peptide is the main constituent of neuritic plaques and derives from the proteolysis of its precursor protein APP. APP is a type I transmembrane glycoprotein that is highly expressed in the central nervous system, where it exerts numerous physiological functions [16]. APP is also widely expressed in normal human tissues, including heart, lung, liver and skin. The APP gene is located on chromosome 21, thus justifying the Aβ overproduction and early development of AD in individuals with Down's syndrome.

APP has a large extracellular domain and a short intracytoplasmatic carboxy-terminus called APP intracellular domain (AICD). As shown in **Figure 3**, APP can undergo cleavage in three different locations: at the N-terminal of the Aβ domain via β-secretase; at the C-terminal of the Aβ domain via γ-secretase; and within the Aβ domain via α-secretase [17].

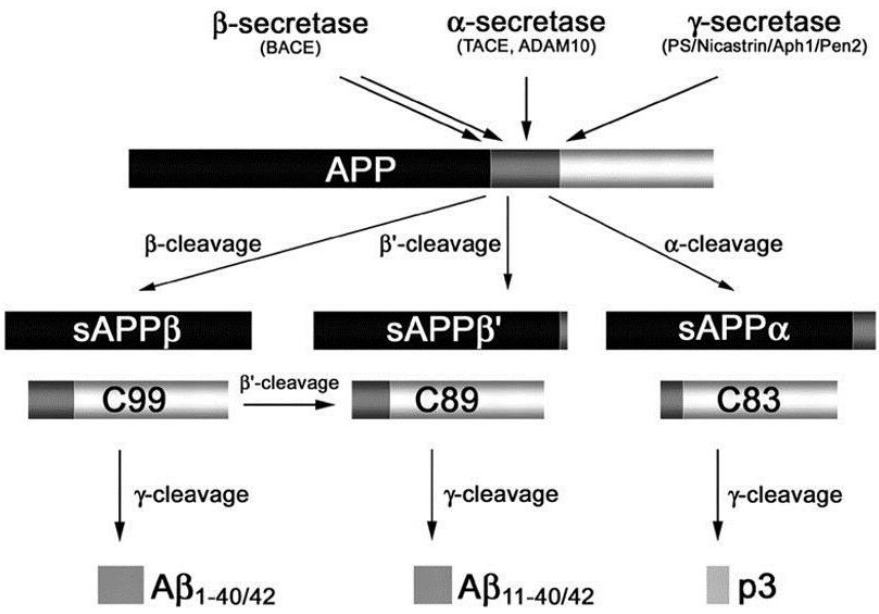

Figure 3 *– The sequential proteolytic processing of amyloid precursor protein*

Under physiological conditions, α-secretase cleaves APP within the Aβ domain at residue L688, generating the soluble extracellular domain of APP (sAPPα) and the membrane-bound carboxy-terminal fragment of 83 amino acids (C83).

The remaining C83 is further processed by γ-secretase to release a soluble P3 peptide and the AICD intracellular domain, both rapidly degraded. This proteolytic pathway precludes the formation of Aβ because α-secretase cuts APP within its amyloidogenic sequence [18].

Alternatively, APP is cleaved by β-secretase, a transmembrane aspartic protease also called β-site APP Cleaving Enzyme (BACE1). BACE1 cleaves APP at Asp1 site to generate C99 (and less frequently at Glu11 site to generate C89) and to release the soluble domain of APP (sAPPβ). C99 (or C89) is then cleaved by γ-

secretase to produce one of several Aβ species, most commonly $A\beta_{1-40}$ and $A\beta_{1-42}$ [17, 19], which are secreted into the extracellular space by exocytosis.

The non-amyloidogenic pathway is particularly active on the plasma membrane [20, 21], where α-secretase is anchored; on the contrary, the amyloidogenic pathway prevails in the endo/lysosomal compartment, where the acidic environment favors BACE1 activity [22, 23].

For long time it was thought that the α-secretase cut had a neuroprotective role, as opposed to the amyloidogenic one operated by BACE1 [24]. However, now we know that the β- and γ-secretase cleavages, respectively at the N- and C- terminal of Aβ, occur in physiological conditions, suggesting that all APP fragments, including Aβ, have a physiological significance [25, 26].

Aβ exists as a monomer, dimer and oligomer, and has a high propensity to form aggregates, such as protofibrils and fibrils, the main constituents of senile plaques in AD brains [27, 28].

Although $A\beta_{1-40}$ is the most abundant form in healthy subjects and AD patients, however, $A\beta_{1-42}$ has aroused huge interest due to its greater tendency to aggregate, forming oligomers with potential neurotoxic activity.

1.1.5. The physiological role of Aβ

Since the discovery of Aβ in 1984 [29], this peptide has been considered the central culprit of AD and, for this reason, the research has mainly been focused on its pathogenic role. However, over the years, there has been a considerable increase in the body of evidence suggesting physiological functions for APP, Aβ and other APP fragments [30].

The first indirect proof of the promnesic role of Aβ dates back to the late 1990s, when it was evident that the intracerebroventricular administration of an anti-APP antibody weakened memory consolidation and recovery [31]. Two years later, Wu and colleagues reported that Aβ increases hippocampal long term potentiation (LTP) [32], while Tamaoka's group demonstrated that $A\beta_{1-40}$ levels in the cerebrospinal fluid (CSF) of AD patients are comparable with those of healthy subjects. Moreover, they also proved that the amount of $A\beta_{1-42}$ (the most neurotoxic species) is significantly lower in the CSF of AD patients, compared to control subjects [33].

Another important evidence supporting the physiological role of Aβ is that APP knockout mice develop age-related cognitive deficits and LTP impairment [34-36], suggestive of a positive effect of Aβ in synaptic plasticity and memory. In line with these observations, Saura and colleagues reported that the loss of presenilin, the enzymatic subunit of γ-secretase, impairs LTP and memory [37].

Upon further investigations, Puzzo and collaborators found that picomolar amounts of $A\beta_{42}$ (similar to those produced physiologically in the healthy brain) enhance hippocampal LTP, while high (nanomolar) concentrations produce opposing results [38]. The same Authors have also shown that either anti-Aβ antibodies or APP knockdown impair LTP and cause cognitive deficits. Even more interestingly, these effects are abolished by intracranial administrations of picomolar $A\beta_{42}$ [39].

The physiological function of Aβ on LTP and memory seems to be mediated by α7 nicotinic receptors (α7-nAchRs) [38, 39]. Indeed, Aβ has picomolar affinity for α7-nAchR [40] and enhances transmitter release in several brain regions including the hippocampus. On the contrary, nanomolar concentrations of Aβ inhibit this

effect by blocking the post-synaptic receptor channel [41]. Accordingly, Puzzo and colleagues showed that Aβ fails to increase LTP in a mouse model where the α7-nAchR is knocked out [38].

A number of studies have also identified other possible targets of Aβ, for example NMDA receptors, RAGE (receptor for advanced glycation end-products), insulin receptor, cellular prion protein, and amylin receptor [42-44].

Considering all the evidences, a novel hypothesis has been proposed, according to which Aβ follows the rule of hormesis, favoring memory at picomolar concentrations and impairing it at higher levels [39, 45, 46]. Under this view, it could be envisaged that if Aβ would somehow lose its functions, a compensatory hyper-production mechanism could occur, leading to the cerebral peptide accumulation.

Certainly, the complete failure of clinical studies aimed at clearing Aβ from the brain [47] is in line with this view.

1.2. Cyclic nucleotides and memory

Memory is the faculty of the brain to acquire, store and consolidate information, and understanding the molecular mechanisms of these processes is one of the main objectives of modern neurosciences.

The second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) are critically involved in the molecular pathways underlying memory formation and have important roles in neuroplasticity, which is generally regarded as the neural correlate of memory, i.e. long-term potentiation (LTP) [48].

Pharmacological and genetic manipulations of the cAMP/PKA/CREB pathway alter the long-lasting form of LTP (L-LTP), which requires protein synthesis, is related to the late memory consolidation process, and is believed to be involved in the long-term memory (LTM) [49, 50].

cGMP has been studied for long time only with respect to the transient first phase of LTP, early-LTP (E-LTP), which is independent from gene expression and is related to the formation of the short term memory [51]. More recently, it has also been implicated in L-LTP and LTM [52, 53].

The increase of cAMP and cGMP by specific phosphodiesterase (PDE) inhibitors (PDE-Is) favors LTP and reduces cognitive deficits in animal models of AD, suggesting PDEs as potential therapeutic targets for the treatment of cognitive dysfunctions [54]. However, the exact role played by cAMP and cGMP in memory processes remains to be elucidated.

1.2.1. cGMP and the mechanism of memory formation

cGMP is produced by guanylate cyclase (GC), which is activated by nitric oxide (NO) [55]. cGMP is quickly degraded by a group of PDEs that catalyze the hydrolysis of cGMP into 5'GMP. There are 11 PDE families and, among them, PDE 5, 6 and 9 are selective for cGMP, whereas PDE 1, 2, 3 and 10 can hydrolyze both cAMP and cGMP.

cGMP activates the cGMP-dependent protein kinase (PKG), which in turn phosphorylates proteins that modulate the synthesis and/or the release of neurotransmitters [56].

In 1998, Son and collaborators indicated cGMP as one of the key intracellular second messengers in the N-methyl-d-aspartate (NMDA) signal transduction pathway that regulates synaptic plasticity [57]. Further studies by Serulle and colleagues demonstrated that the increase of cGMP activates signaling mediators such as the GMP-dependent protein kinase II (cGKII), which phosphorylates GluR1, an important promotor of synaptic plasticity [58].

In line with these studies, increasing cGMP levels by using PDE9 inhibitors improved LTP in hippocampal cultured neurons [59] and slices [60, 61].

The memory-improving efficacy of PDE9 inhibitors has been proved *in vivo* using behavioral paradigms such as T-maze [62], object location [61] and novel object recognition test [59], in animal models.

In humans, also because AD patients show reduced CSF levels of cGMP [63], inhibiting PDEs has been suggested as a possible strategy to ameliorate cognitive and memory deficits [64, 65].

Despite the fact that it is not exactly clear how PDE-Is influence the processes of learning and memory *in vivo*, very recent findings indicate that Aβ is one of the downstream effectors for cAMP and cGMP to trigger synaptic plasticity [65, 66].

1.2.2. The cGMP-Aβ correlation in cognitive processes

Since cAMP, cGMP and Aβ peptides have been involved in the sustaining of LTP and memory, it became crucial to understand whether these three components are connected or acting independently from each other.

Following this line of research, the lab where I worked at this thesis demonstrated that cAMP exerts positive effects on LTP through the stimulation of APP synthesis and Aβ production [67, 68].

Subsequently, also cGMP was proven to increase Aβ, but through a different mechanism: stimulating the convergence of APP and BACE-1 in endo-lysosomal compartments where the amyloidogenic processing of APP is favored [50, 65].

As previously observed with cAMP, cGMP could not support hippocampal LTP when specific anti-Aβ antibodies or APP^{KO} mice were used, but as soon as picomolar concentrations of synthetic Aβ were supplied, the LTP could be restored [65].

In **Figure 4** is represented the theoretical model explaining how cGMP and cAMP modulate Aβ leading to the enhancement of LTP [66].

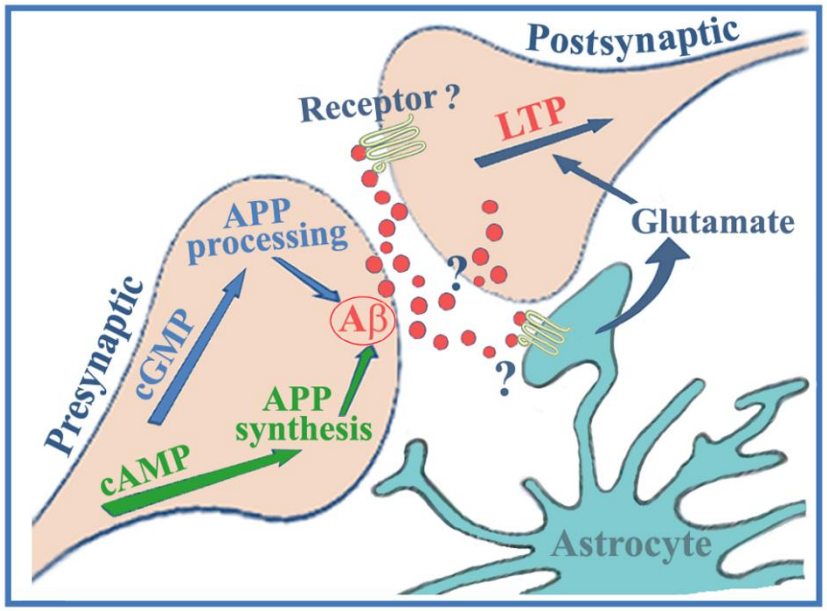

Figure 4 *– Theoretical model explaining how cGMP and cAMP modulate Aβ leading to the enhancement of LTP. At presynaptic region, cAMP (green) stimulates Aβ production by inducing APP synthesis, whereas cGMP (blue) increases Aβ levels by modulating the processing of APP. In particular, cGMP favors the approximation of APP and BACE-1 in endo-lysosomal compartments. Once secreted, Aβ (red) might influence LTP by activating postsynaptic receptors and/or the astrocytic α7 nicotinic acetylcholine receptor. It is also possible that Aβ may act by entering the postsynaptic intracellular compartment.*

1.3. Endocytic mechanisms

Endocytosis is an important cellular mechanism by which cells generate small (60-120 nm) membrane vesicles used to transport various cargo molecules, extracellular ligands, membrane proteins and lipids from the cell surface to the internal compartments [69, 70].

Multiple mechanisms of endocytosis occur in eukaryotic cells and they are divided into two broad categories: phagocytosis or cell eating (the uptake of large particles) and pinocytosis or cell drinking (the uptake of fluid and solutes). Phagocytosis is the function of specialized cells (macrophages, neutrophils and monocytes) of ingesting and clearing foreign materials, such as pathogens or large debris. Pinocytosis, as shown in the **Figure 5,** includes four mechanisms: macropinocytosis, clathrin-mediated endocytosis (CME) [71], caveolae-mediated endocytosis, and clathrin- and caveolae- independent endocytosis [72].

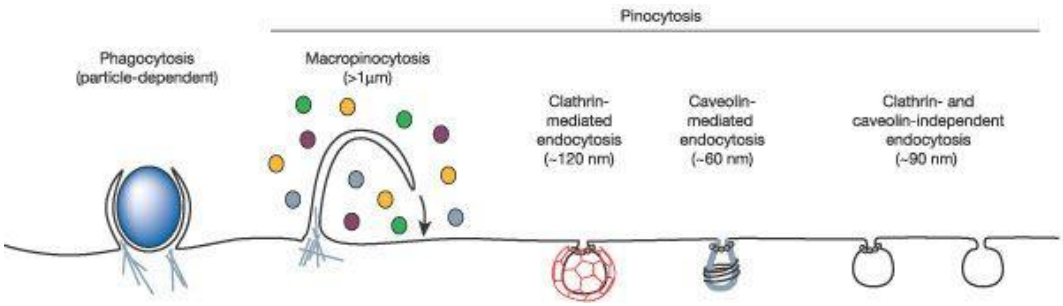

***Figure 5** – Different processes of entry into the cell. These mechanisms differ from each other according to the size of the endocytic vesicle, the nature of the cargo and the mechanism of vesicle formation.*

Despite different endocytic pathways have been described, CME is the major route for the internalization of many cargoes and it is crucial for intercellular communication.

Clathrin was first described in the 1960s as a regular protein coating the membrane of endocytic pits and vesicles in mosquito oocytes [73]. Clathrin is composed of three heavy chains, each of them binding a light chain to form a three-legged structure named triskelion. When the triskelia interact they assemble into a polygonal lattice, which helps to deform the overlying plasma membrane into a coated pit [70, 74].

Whatever the entry mechanism is, the cargo molecules are captured in the endosomes that are pinched off from the plasma membrane and then fuse with early or sorting endosome moving to final destination [69] .

There are different types of endosomes and they can be designed as incoming endosomes, early endosomes, late endosomes or recycling endosomes, depending on the kinetics by which they are loaded with cargo and also with respect to their morphology [75]. Once endocytic vesicles have uncoated, they fuse with early endosomes, and then they mature into late endosomes before fusing with lysosomes for the cargo degradation [76].

The formation of transport vesicles and their specific delivery to target membranes are often regulated by small G proteins of the Rab and Arf families [69].

1.3.1. The role of Rab GTPases in regulating endocytosis

Rab proteins are small GTPases that belong to the Ras superfamily and regulate the vesicular transport in endocytosis and exocytosis. There are over 70 human Rab GTPases and more than half of them are involved in regulating ensosomal membrane traffic [77, 78].

Rab GTPases define compartment identity and are principally implicated in vesicle formation, transport, docking and fusion to the target compartment. These different roles are supported by many Rab effector proteins [69].

The ubiquitous Rab GTPases Rab5, Rab4 and Rab11 function on the early endocytic pathway, whereas Rab7 and Rab9 function on the late endocytic pathway. Rab5 can also be detected at the plasma membrane [79].

Rab proteins are recruited to and activated on the donor membrane, where they are important in vesicle budding. In particular, activated Rab5 is involved in sequestering ligands into clathrin coated vesicles and in the subsequent vesicle fusion with early endosomes [80]. Rab7 acts downstream from Rab5 and regulates the transport from early to late endosomes and lysosomes, whereas Rab4 and Rab11 regulate the transport along the recycling pathway, from early and recycling endosomes to the cell surface [78].

Live-cell imaging studies conducted by Rink and colleagues have demonstrated that Rab5 levels at the endosomal membrane are not stable, but they fluctuate dynamically. Through repetitive fusion and fission events, the cargo into early endosomes becomes concentrated, while endosomes enlarge. On these large endosomes there is a complete loss of Rab5, which is replaced by Rab7. This conversion mechanism from Rab5 to Rab7 marks the transition of cargo from early to late endosomes.

Rab proteins function in sequence and are activated and inactivated in a "cascade-like" manner [81].

1.3.2. The Rab activation cycle

Rab proteins cycle between the cytosol and the membrane of their respective transport compartment [82].

After translation, Rab is first associated with a Rab escort protein, which allows the C-terminal modification of Rab by the addition of one or two geranylgeranyl lipid groups. This change favors the Rab-membrane association when Rab is in the GTP-bound state (active state). A protein named GDP displacement factor (GDF) inserts Rab in the appropriate membrane, whereas a GDP dissociation inhibitor (GDI) mediates the membrane extraction of inactive Rab and leads to the formation of a cytosolic complex [83]. **Figure 6** schematically illustrates this cycle: after membrane binding of Rab, a guanine nucleotide exchange factor (GEF) promotes the release of GDP and the subsequent loading of GTP. In its GTP-bound conformation, Rab is active and able to interact with specific effectors that, in turn, may trigger events such as vesicle fusion with a target membrane. To complete the cycle, a GTPase activating protein (GAP) binds to Rab and catalyzes the nucleotide hydrolysis, switching off the GTPase. The remaining GDP-bound Rab can then participate in a new round of fusion [77, 82].

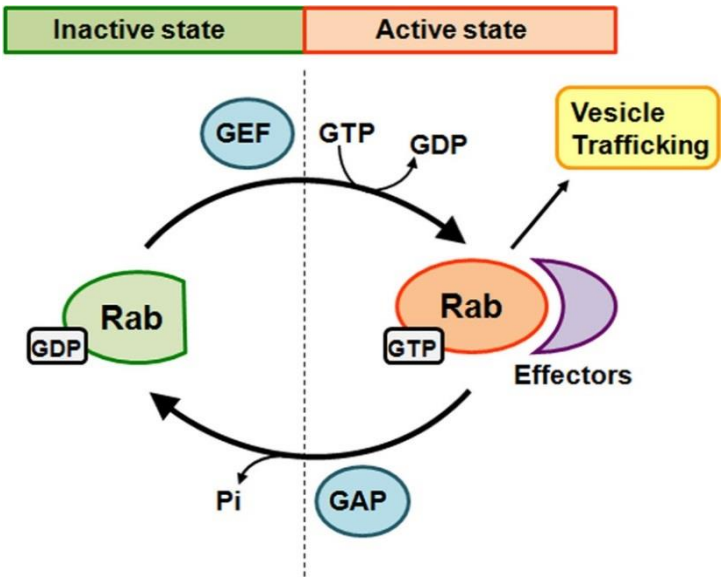

Figure 6 *– The Rab GTPase cycle. Rab proteins switch between two conformations: an inactive GDP-bound form and an active GTP-bound form. GEF allows the activation of Rabs by catalyzing the conversion from the GDP-bound to the GTP-bound form. After binding to specific effectors, active Rab promotes vesicle trafficking. GAP leads to Rab inactivation by hydrolyzing the bound GTP to GDP.*

1.3.3. The role of Rabs in Alzheimer's disease

Rab proteins have been implicated in the trafficking of AD-related proteins; moreover, expression of certain Rab members was found to be affected in AD brains. These alterations have been related to endo-lysosomal dysregulation that may contribute to AD pathogenesis.

In fact, enlargement of Rab5-positive early endosomes has been observed in the brain of AD patients together with the upregulation of Rab4, Rab5, Rab7, Rab10 and Rab27 in cholinergic basal forebrain neurons and CA1 pyramidal neurons [84, 85].

Another piece of evidence indicates that RIN3, a Rab5-GEF, is a risk factor for AD [86], whereas studies conducted by Ridge and collaborators suggest that the downregulation of Rab10 modulates $A\beta_{42}$ levels and $A\beta_{42}/A\beta_{40}$ ratio in neuroblastoma cells [87].

Grbovic's results have shown that Rab5 overexpression mimics the morphological changes in early endosomes seen in neurons from AD brains and demonstrated that the overactivation of this GTPase increases both $A\beta_{40}$ and $A\beta_{42}$ secretion in conditioned medium from murine fibroblast-like L cells stably transfected with human APP_{695} (L/APP) [88]. Accordingly, Xu reported an abnormal activation of Rab5 in post-mortem brain samples of AD patients and AD mouse models [89, 90]. Moreover, Kim and colleagues demonstrated that elevated levels of the β-cleaved carboxy-terminal fragment of APP (β-CTF) induce Rab5 overactivation by recruiting APPL1, a protein that stabilizes active GTP-Rab5 leading to pathologically accelerated endocytosis [91].

In the light of current knowledge, it is likely to assume that alterations of Rab family members are somehow related to aberrant trafficking, signaling, and ultimately neurodegeneration, throughout the progression of AD.

1.3.4. ADP-ribosylation factor proteins (Arfs)

Arf proteins belong to a group of six small (20 kDa) GTPases. Like Rabs, they are related to the Ras family and their main function is that of maintaining the integrity of intracellular transport [92].

Arf and Rab share a similar activation cycle, which involves GEFs and GAP proteins.

Three classes of Arf proteins are expressed in mammals: class I (including Arf 1, 2 and 3), class II (including Arf 4 and 5), and class III (including only Arf 6) [93]. Arfs localize to the plasma membrane and to membranes of secretory, endosomal, and lysosomal pathways. Differently from the other Arf proteins, Arf 6 has no effect on Golgi membrane dynamics. It regulates the cortical actin cytoskeleton, the endosomal trafficking [69, 93], and the CME, [94, 95], although an alternative role of Arf 6 in clathrin- and caveolae- independent internalization mechanisms has been suggested [96].

1.3.5. APP and BACE1 trafficking

After protein synthesis in the endoplasmic reticulum, both APP and BACE1 mature through the constitutive secretory pathway.

Most of APP is found in the Golgi complex and trans-Golgi network and only a small portion is detected at the cell surface. In fact, over 50% of mature APP is internalized within 10 minutes and sorted into early endosomes. Here, one fraction is recycled back to the plasma membrane and another fraction is targeted to lysosomes for degradation.

BACE1 is also localized in the trans-Golgi network and endosomes and is rapidly internalized from the cell surface [97, 98]. However, APP and BACE1 do not co-localize at the plasma membrane, where APP is generally cleaved by α-secretase. Rab5-positive early endosomes are the main compartments where BACE1 encounters APP. In fact, the endosomal acidic environment is optimal for BACE1 activity [97].

Recently, Sannerud and collaborators demonstrated that the internalization of BACE1 is independent of clathrin and requires Arf6 activity [99]. Differently, the trafficking of APP from the cell surface to Rab5-positive early endosome occurs via clathrin-dependent endocytosis (**Figure 7**) [97, 100].

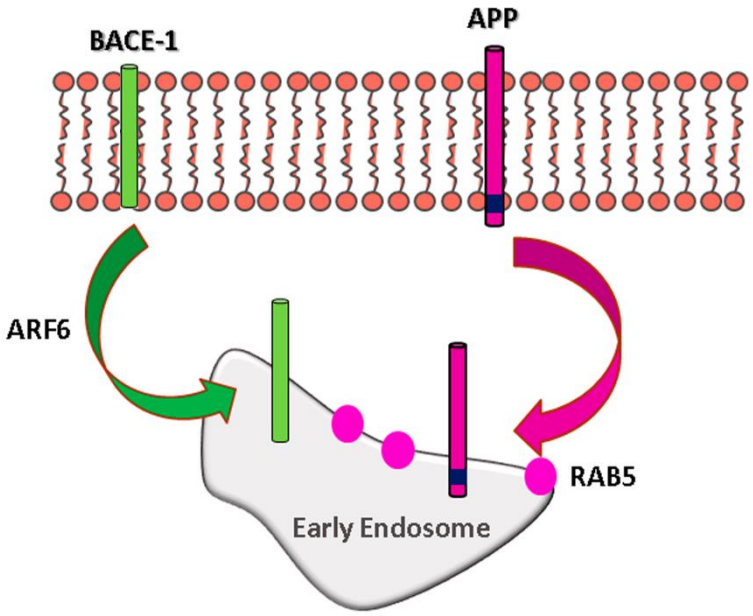

Figure 7 *– Schematic view of APP and BACE1 internalization. Under normal conditions, early endosomes marked by Rab5 are the main sites of APP processing by BACE1. APP undergoes internalization from the plasma membrane to early endosomes within clathrin-coated vesicles, whereas BACE1 is sorted to early endosomes through a clathrin-independent route that is controlled by Arf6.*

Chapter 2

Aims

Previous data from the laboratory where I carried out this study demonstrated that the enhancement of cyclic guanosine monophosphate (cGMP) in neuronal microdomains triggers the interaction of APP and BACE1 in early endosomes. As a consequence, this induced the production of Aβ peptides, which was instrumental to sustain hippocampal long-term potentiation and memory formation [65].

In light of these findings, the present study aimed to better characterize the trafficking of APP and BACE1 under cGMP stimulation in order to provide new molecular details that may improve our understanding of AD pathogenesis.

As already mentioned in the Introduction, overactivation of Rab5 has been shown to cause endosome enlargement, one of the earliest pathological alterations observed in the brain of AD and Down syndrome patients [89]; furthermore, expression of a dominant-negative Rab5 mutant was found to reverse neuronal atrophy in *Droshophila* [90].

Although early endosomes marked by this small GTPase represent the major site of APP processing by BACE1 [101, 102], the mechanism by which APP meets BACE1 in Rab5-positive endosomes is still unknown.

The first objective of my research was to investigate whether cGMP plays a role in the internalization of APP from the cell surface to the endosomal compartment, thus explaining, at least in part, how the cyclic nucleotide stimulates APP to interact with BACE1 in early endosomes.

Thereafter, we investigated whether Rab5 and/or Arf6 may mediate the effects induced by the cyclic nucleotide on the trafficking and the amyloidogenic processing of APP.

Chapter 3

Results

3.1. cGMP stimulates the internalization of APP

Previous results from our laboratory demonstrated an increased co-localization of APP and BACE1 in primary neurons treated with vardenafil, an inhibitor of PDE5 that enhances intracellular cGMP [65]. In particular, this co-localization was found to take place in endolysosomal vesicles, compartments where the activation of BACE1 is favored by the acidic pH [50].

Here, we first tested whether cGMP could somehow trigger the endocytosis of APP.

To this aim, we used N2a cells overexpressing APP, as only a small fraction of the protein is normally present at the plasma membrane. Cells were exposed to vardenafil for different times (0-60 min) and then processed for cell surface protein biotinylation. Immunoblot analysis of the biotin-labeled proteins revealed that the amount of plasma membrane-bound APP decreases in a time-dependent manner after vardenafil treatment (-34% at 10 min-exposure, $P < 0.01$; -39% at 30 min, $P < 0.01$; -46% at 60 min, $P < 0.001$) (**Figure 8**).

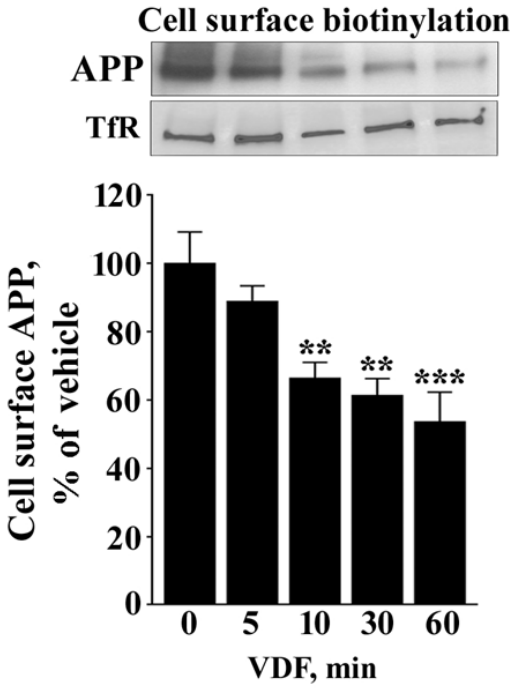

Figure 8 *– cGMP induces a faster APP internalization. N2aAPP cells were treated with 100 μM vardenafil (VDF) for the indicated time periods. Control samples (0 min) received the same volume of vehicle (DMSO) for 60 min. After cell surface biotinylation, followed by cell lysis and precipitation with NeutrAvidin™ -beads, levels of APP in the plasma membrane were determined by immunoblot analysis with 22C11 antibody. The transferrin receptor (TfR) was used as a loading control. Results are expressed as mean ± SEM for at least four independent experiments. **P < 0.005 and ***P < 0.0001 vs. vehicle-treated group (one-way ANOVA, Dunnet post-test).*

In order to verify whether the reduction of APP on the cell surface may reflect its increased internalization, we performed a surface APP immunostaining on ice, without permeabilization [103], before incubating the cells with vardenafil for 10 sec or 10 min at 37 °C. Using confocal microscopy analysis, we found that 10 sec after treatment, as expected, APP immunoreactivity was located on the surface of

both vardenafil and control cells (**Figure 9**, upper panels). At 10 min exposure, control cells showed an APP-associated fluorescence still located at or just beneath the plasma membrane, whereas a pronounced translocation of APP to intracellular vesicle-like puncta was observed in vardenafil treated cells (**Figure 9**, lower panels), strongly suggesting a faster endocytosis of the protein.

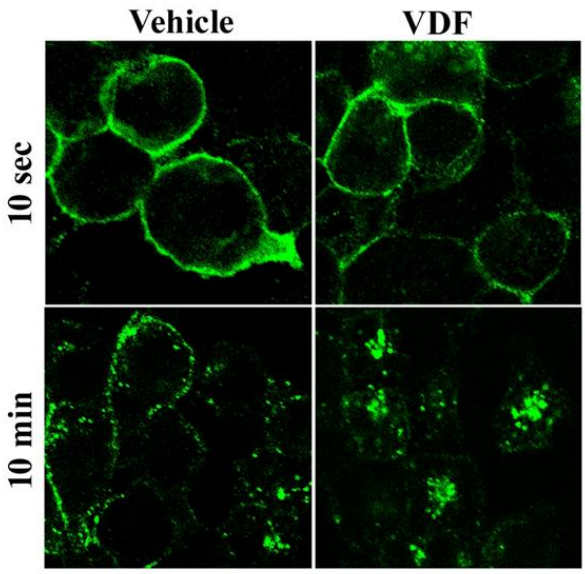

Figure 9 *– Confocal images of N2aAPP cells incubated at 4 °C with the anti-APP antibody 6E10, as described in Materials and Methods, and then treated with 100 μM vardenafil (VDF) or DMSO (vehicle) for 10 sec or 10 min at 37 °C. Cells were then fixed, permeabilized and labeled with a green fluorescent secondary antibody. Figure is representative of 3 independent experiments with essentially similar results.*

3.2. Inhibition of endocytosis prevents the amyloidogenic effect of cGMP

Next, we investigated the impact of endocytosis on the cGMP-induced amyloidogenesis. To this aim, N2a cells were pre-treated with PitStop2, an inhibitor of both clathrin-dependent and clathrin-independent endocytic pathways [104], and then exposed to the cGMP-enhancer vardenafil. As expected, the inhibition of endocytosis *per se* reduced the production of Aβ peptides [105] (67% of control, $P < 0.05$), whereas vardenafil robustly increased it [50] (286% of control, $P < 0.0001$). Notably, the effect of vardenafil was totally prevented by PitStop2, indicating that the endocytic process is required for cGMP to increase Aβ production (**Figure 10**).

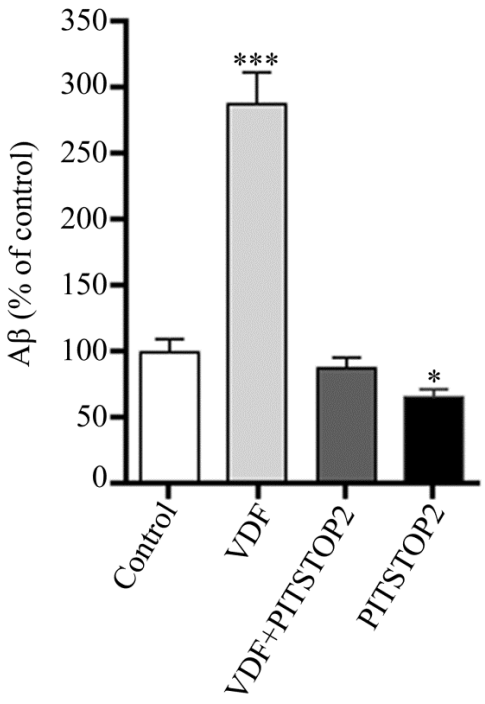

Figure 10 *– The production of Aβ induced by cGMP is prevented by the inhibition of endocytosis. Where indicated, N2a cells were pretreated with 25 μM PITSTOP2 for 10 min and then incubated for 1 h with 100 μM vardenafil (VDF) or an equal volume of vehicle (Control). At the end of the incubation period, conditioned media were subjected to specific $Aβ_{42}$ -ELISA. Graphed data show mean ± SEM for at least three independent experiments. ***P < 0.0001; *P < 0.05 vs vehicle-treated group.*

3.3. Rab5 activation state modulates Aβ production

Given the involvement of Rab5 [89] and Arf6 [99] in APP and BACE1 endosomal trafficking, respectively, we evaluated the amount of Aβ peptides in conditioned media of cells transiently transfected with mRFP-Rab5^{WT}, HA-Arf6^{WT}, or their constitutively active (mCherry-Rab5^{CA}, HA-Arf6^{CA}) and dominant-negative (mCherry-Rab5^{DN}, HA-Arf6^{DN}) mutants. Efficiency of transfections in multiple experiments was assessed by immunoblot analyses, and typical levels of expression are shown in **Figure 11A**. Overexpression of either WT or mutant forms of HA-Arf6 did not alter the amount of Aβ released by the cells in the culture media, and similar results were obtained in mRFP-Rab5^{WT} expressing samples (**Figure 11B**). On the contrary, overexpression of mCherry-Rab5^{DN} increased the Aβ release (130% of control, $P < 0.05$), while a slight, but statistically significant decrease was observed in culture media of cells transfected with mCherry-Rab5^{CA} (83% of control, $P < 0.01$) (**Figure 11B**).

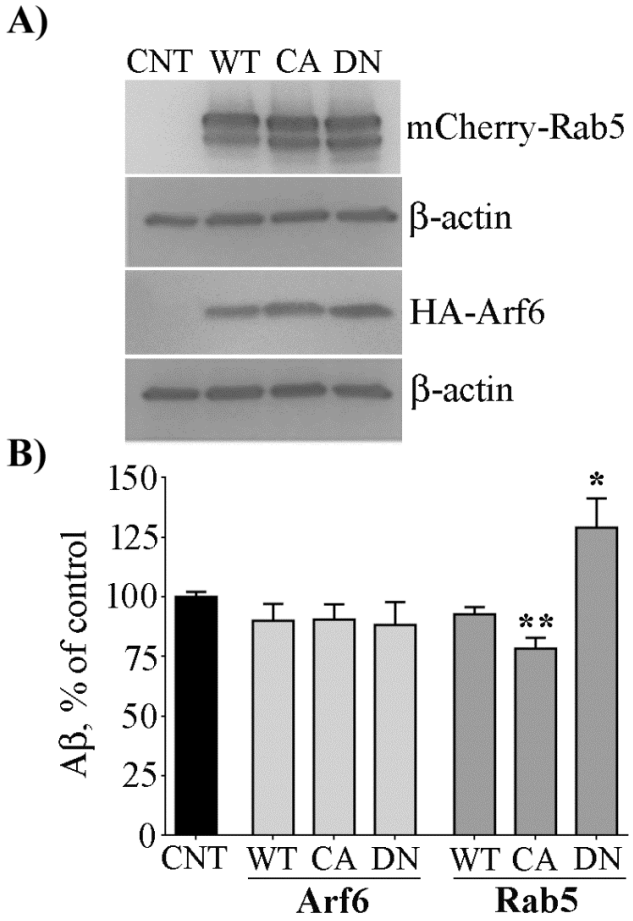

Figure 11 *– Rab5 but not Arf6 mutations influence Aβ production. Where indicated, N2a cells were transfected with HA-Arf6^{WT}, HA-Arf6^{CA}, HA-Arf6^{DN}, or with mRFP-Rab5^{WT}, mCherry-Rab5^{CA}, and mCherry-Rab5^{DN}. After 24 h, cells were processed for total protein extraction, and immunoblot analyses were performed with anti-Rab5 and anti-HA antibodies to verify the efficiency of transfections* ***(A)****, while conditioned media were subjected to specific $A\beta_{42}$ -ELISA* ***(B)****. The β-actin signal represents the internal loading control. Graphed data show mean ± SEM for at least three independent experiments. *P < 0.05; **P < 0.005 vs control group.*

3.4 Rab5 siRNA increases Aβ peptides

In order to confirm the correlation between Rab5 and Aβ, we induced Rab5 transient knockdown in N2a cells using specific siRNAs. Forty-eight hours after siRNA transfections, conditioned media were subjected to Aβ ELISA, whereas cell extracts were analyzed for Rab5 expression by immunoblot. In line with the effect exerted by $Rab5^{DN}$, silencing of Rab5 significantly increased the amount of Aβ released by the cells in the culture medium (131% of non-targeting control siRNA, $P < 0.05$).

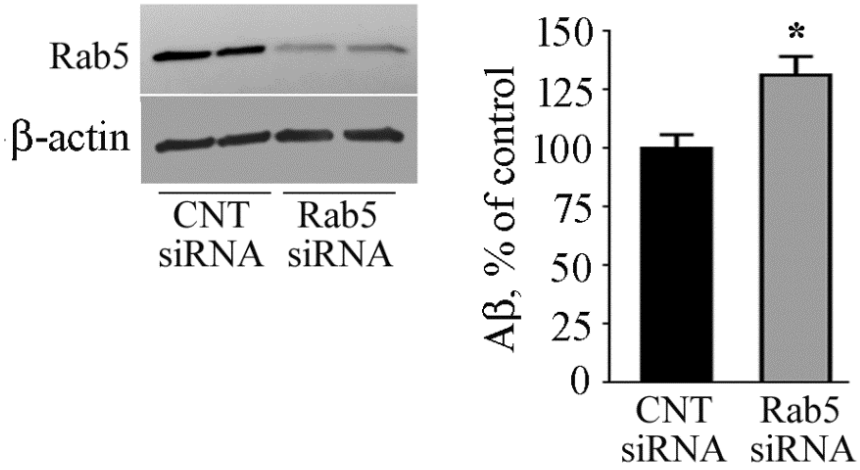

Figure 12 *– Rab5 siRNA increases Aβ production. N2a cells were transfected with Rab5 siRNA or non- targeting siRNA (CNT siRNA). After 48 h, media were changed and collected 1 h later for $Aβ_{42}$-ELISA. At the same time, cells were processed for Rab5 immunoblotting. The β-actin signal represents the internal loading control. Graphed data show mean ± SEM for at least three independent experiments. *P < 0.05.*

3.5. cGMP reduces GTP-Rab5 levels

We next sought to examine whether an increase of intracellular cGMP could influence the activation of Rab5. As a small GTPase, Rab5 cycles between a GDP- (inactive) and GTP-bound form (active) [106]. Therefore, we measured GTP-Rab5 levels in cells exposed to vardenafil for different times (5, 10, 30 and 60 min). As shown in **Figure 13**, an approximately 50% reduction of active GTP-bound Rab5 was already evident, although not statistically significant, after 5 min of vardenafil treatment ($P = 0.088$ vs control). The amount of GTP-Rab5 dropped to 40% of control after 10 min ($P < 0.01$) and remained depressed after 30 and 60 min of treatment (45% of control, $P < 0.05$, at both time points). Because the total expression of Rab5 did not change at any time of vardenafil exposure (**Figure 13**, Total Rab5 panel), it is likely to assume that cGMP increased the inactive GDP-bound form of the small GTPase.

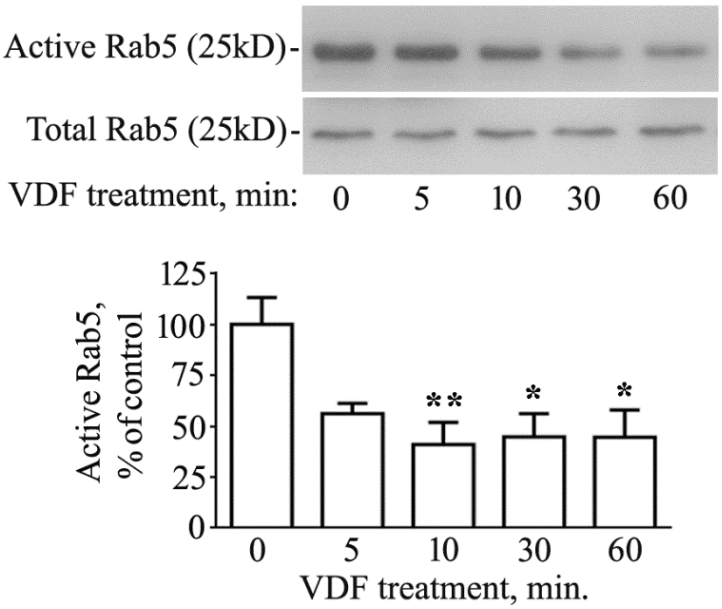

Figure 13 *– cGMP inhibits Rab5 activity. N2a cells were treated with 100 μM vardenafil (VDF) for different times (5, 10, 30, 60 min). Control samples (0 min) received the same volume of vehicle (DMSO) for 60 min. At the end of treatments, cells were processed for total protein extraction followed by Rab5 activity assay, as described in the Material and Methods section. Top, representative immunoblots; bottom, graphed data showing mean ± SEM for at least three independent experiments. *P < 0.05; **P < 0.005 vs vehicle-treated group.*

3.6. cGMP decreases Rab5 activity without modifying full-length APP or CTFs expression

Recently, Kim and collaborators shown that β-CTF, the APP soluble fragment generated by BACE1, is able to affect Rab5 activity [91]. To investigate whether, in our conditions, the inhibition of Rab5 could be due to the increase of β-CTF levels, we analyzed the APP fragments produced by the cells after vardenafil treatments. β-CTFs are rapidly cleaved by γ-secretase to generate Aβ peptides, therefore, to allow their detection, we pre-treated the cells with 2 μM DAPT, a γ-secretase inhibitor.

As shown in **Figure 14**, vardenafil did not modify the levels of full length APP or those of C99 and C89, the main β-CTFs. Moreover, also the C83 fragments generated by α-secretase were not affected by the cell treatment.

These data indicate that the effect exerted by cGMP on the activation state of Rab5 is not mediated by the products of APP cleavage. The possibility that Aβ peptides could indeed play a role in the observed phenomenon was already excluded by others [90].

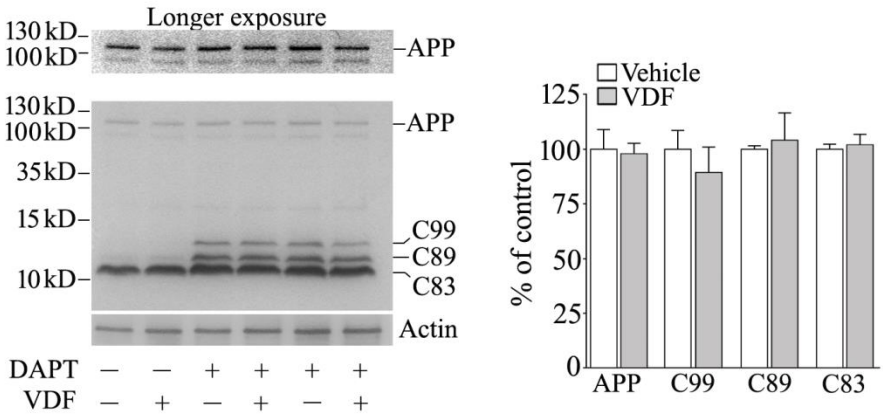

Figure 14 *– cGMP does not affect APP and its fragments. Expression of APP and CTFs in N2aAPP cell treated with vardenafil (VDF). Left, representative immunoblot analysis performed with an anti-APP-CTF antibody; right, graphed data showing mean ± SEM for at least three independent experiments. The β-actin signal represents the internal loading control.*

3.7. Rab5^{DN} mutant stimulates APP and BACE1 interaction

Using the Optical Convergence of APP and BACE1 (OptiCAB) assay [107], previous studies from our laboratory demonstrated that cGMP stimulates the interaction between APP and BACE1 [65]. Here, given the effect of vardenafil on the activation state of Rab5, we took advantage of the OptiCAB assay to investigate whether the overexpression of Rab5^{DN}, which has a much higher affinity for GDP compared to GTP, could modify the approximation between APP and BACE1. To this aim, 16 hours after mCherry-Rab5^{DN} transfection, N2a cells were further transfected with APP tagged with the N-terminal fragment of the Venus fluorescent protein (APP:VN) and with BACE1 tagged with the complementary C-terminus (BACE1:VC). In this manner, the physical approximation of APP:VN and BACE1:VC allows the reconstitution of Venus protein, which becomes fluorescent (**Figure 15**).

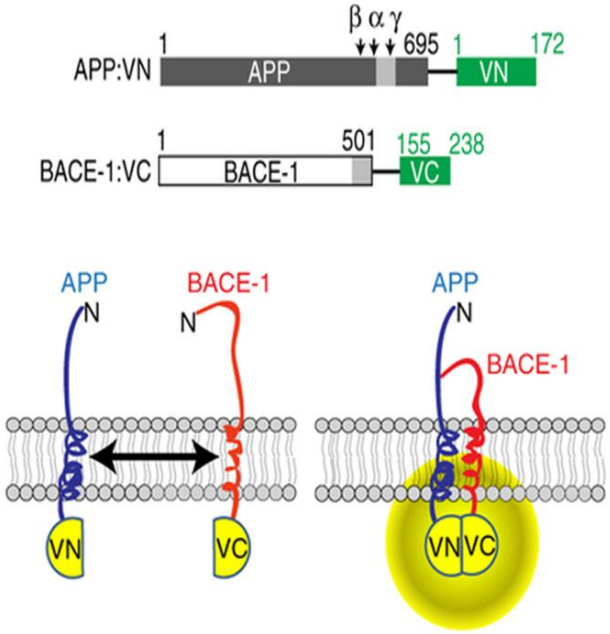

Figure 15– *OptiCAB assay. APP and BACE1 are respectively tagged with the N-terminal (VN) and the C-terminal (VC) portion of Venus protein. When APP interacts with BACE1, Venus fluorescence is reconstituted.*

Confocal microscopy analysis showed that the expression of $Rab5^{DN}$ is able to increase APP-BACE1 approximation (**Figure 16**), which is suggestive of an increased interaction between BACE1 and its substrate. As a matter of fact, this effect exactly resembled that induced by the cGMP-enhancer vardenafil. Accordingly, in cells overexpressing $Rab5^{CA}$, APP and BACE1 showed a reduced interaction, as indicated by an almost total absence of green fluorescence (**Figure 16**).

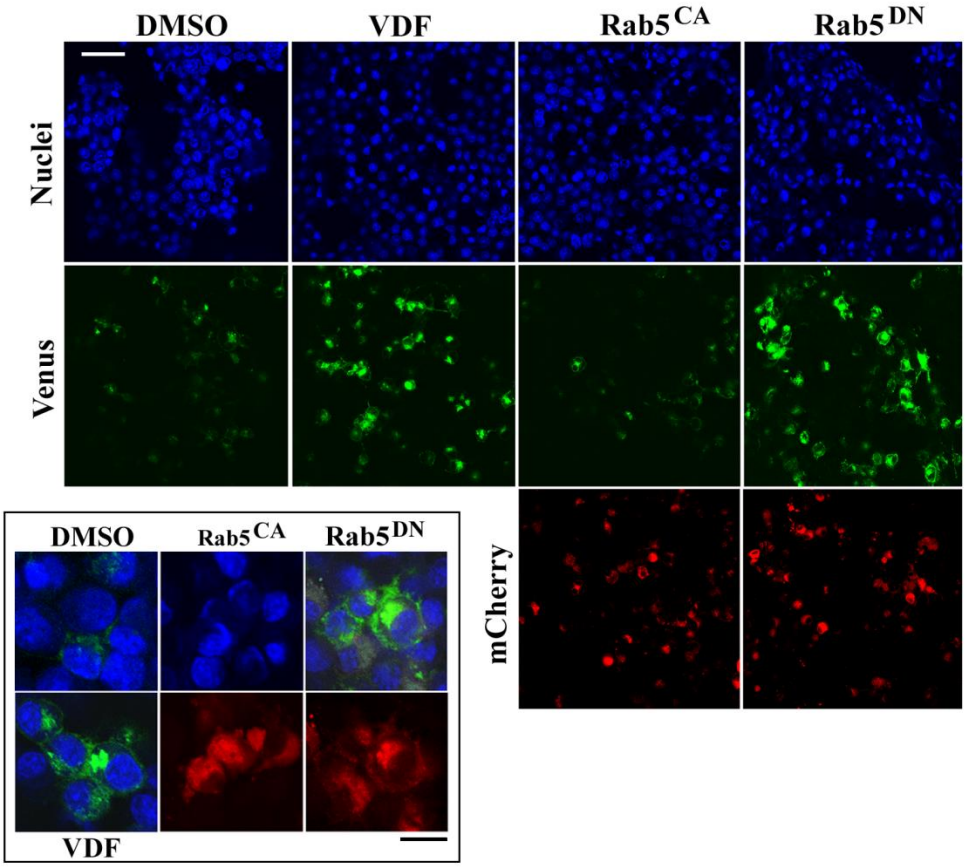

Figure 16 *– Rab5^{DN} mutant triggers the interaction between APP and BACE1. For confocal analyses, N2a cells were transfected with APP:VN and BACE1:VC expressing vectors. Only where indicated, cells additionally expressed mCherry-Rab5^{DN} (Rab5^{DN}), mCherry-Rab5^{CA} (Rab5^{CA}), or underwent vardenafil treatment (VDF). Nuclei are blue-fluorescent, the green fluorescence is indicative of APP-BACE1 interaction, whereas the red fluorescence is due to the expression of mutant mCherry-Rab5. Note the increase in the APP-BACE1 interaction in cells treated with vardenafil, compared to control cells, and how this effect is mimicked by Rab5^{DN} but not Rab5^{CA} expression. The inset shows merged blue/green fluorescence, and mCherryRab5 expression, at a great magnification. White scale bar = 50 μm; black scale bar = 20 μm. Figure is representative of 3 independent experiments, all showing essentially similar results.*

Chapter 4

Discussion and Conclusion

The laboratory where I worked at this thesis demonstrated that cGMP stimulates the approximation of APP and BACE1 in endolysosomal compartments, consequently increasing the production of Aβ peptides [65].

To further investigate this issue, the project of my PhD focused on the identification of the mechanism by which cGMP regulates the subcellular co-localization of APP and BACE1.

Firstly, we provided evidence that cGMP has a role in the endocytic pathway of APP. In fact, our results indicate that cGMP is able to speed up the internalization of APP from the plasma membrane to endosomal compartments where BACE1 is preferentially active. Moreover, we found that the amyloidogenic effect of cGMP is abolished by PitStop2, an inhibitor of both clathrin-dependent and clathrin-independent endocytic pathways, indicating that endocytosis is necessary for cGMP to increase Aβ.

Secondly, we investigated the involvement of two GTPases, Rab5 and Arf6, respectively implicated in APP and BACE1 endocytic sorting. Our findings demonstrated that Rab5, but not Arf6, has an impact on the production of Aβ peptides. Specifically, we found that expression of a dominant mutant that keeps Rab5 in its inactive (GDP-bound) form increases the amount of Aβ peptides released by the cells, thus mimicking the effect of cGMP [65]. Moreover, Aβ levels are increased when Rab5 expression is knocked down by siRNA.

This result prompted us to investigate whether cGMP could influence the activation state of Rab5, consequently leading to the observation that the cyclic nucleotide maintains Rab5 in its GDP-bound conformation.

Finally, we found that expression of the inactive Rab5 mutant increases APP-BACE1 interaction, an effect previously detected in primary neurons treated with vardenafil [65], and confirmed in the present study.

Taken together, these results suggest that the well-established positive effects exerted by cGMP on synaptic plasticity and memory formation [66] may require low Rab5-GTP levels, thus explaining, at least in part, why the upregulation of the small GTPase is associated with neurodegenerative phenomena [89]. Consistent with this hypothesis, a new transgenic mouse model of neuronal Rab5 over-activation was very recently shown to develop AD-related endosome dysfunction and AD-like deficits in axonal transport, synaptic plasticity, cognition, and neuronal survival [108].

Another important evidence in the present study is that the inactivation of Rab5 correlates with an increased production of Aβ peptides, an event that, based on the "amyloid hypotesis" of AD [11], would generate detrimental consequences. Indeed, our data fit well with the notion that Aβ boosts hippocampal activity by regulating synaptic vesicle release [109], and with the studies of Puzzo and collaborators, who clearly demonstrated that physiological (picomolar) concentrations of $A\beta_{42}$ are required to sustain LTP and cognitive performance [38, 39].

On the other hand, our findings seem at variance with previous reports showing that overexpression of either wild-type Rab5 [88], or dominant negative Arf6-T27N increases the production of Aβ peptides. It should be noted, however, that those studies have been performed in non-neuronal cells engineered to overproduce Aβ, thus under different conditions that might justify the different results. In fact, as far as we know, there is no evidence of an increased Aβ

production in mice expressing over-activated neuronal Rab5, despite the occurrence of AD-like deficits [108].

Collectively, our data support a model in which cGMP favors the endosomal interaction between APP and BACE1 by keeping Rab5 in its GDP-bound conformation, consequently leading to the production of Aβ peptides that, in turn, sustain LTP and memory formation/consolidation (**Figure 16**).

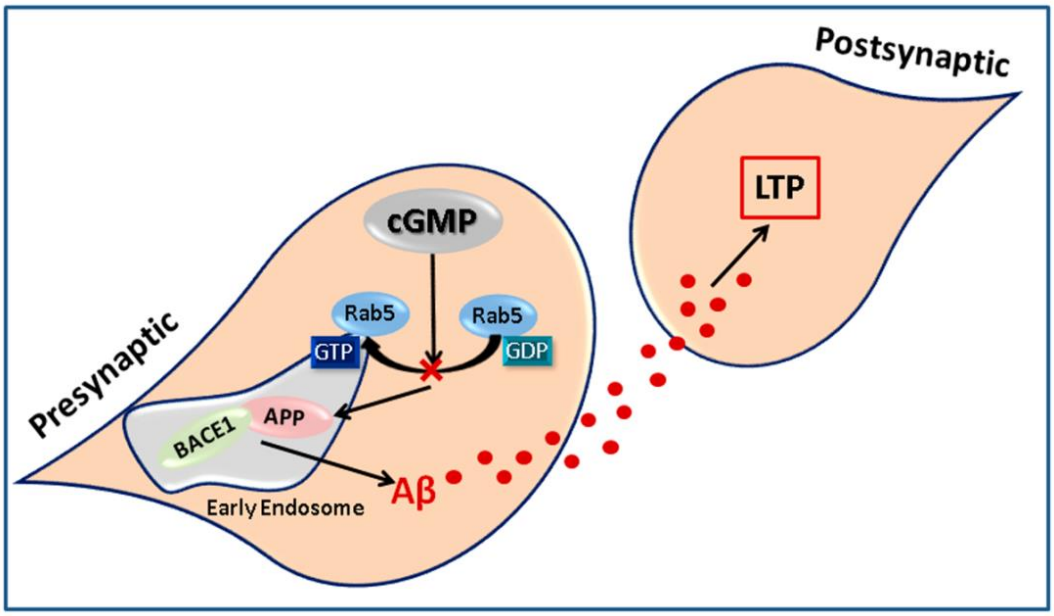

Figure 16 *– Theoretical model illustrating the cGMP-LTP pathway. At presynaptic level, the enhancement of cGMP keeps Rab5 in its GDP-bound conformation, thus decreasing GTP-Rab5 levels and allowing APP to interact with BACE1 in early endosomal compartments. This process leads to the production of Aβ peptides that, in turn, sustain postsynaptic LTP.*

It remains to be clarified how cGMP preserves the GDP-bound conformation of Rab5. In this respect, it can be hypothesized an involvement of Rab5-GAP, the protein that hydrolyses GTP to GDP, or Rab5-GEF, which is known to activate Rab5 [83]. Furthermore, an effect of cGMP on the activity of other Rab proteins cannot be excluded at this stage of the study. Rab7 and Rab4, for example, have been involved in the maturation of early endosomes and implicated in the recycling of APP from early endosomes to the plasma membrane [81, 110].

Another possibility is that suggested by Bucci and collaborators, who demonstrated that cells overexpressing dominant inactive Rab5 are characterized by smaller endocytic structures [106]. Accordingly, hyperactivation of Rab5 has been associated with enlarged early endosomes, an abnormality visible in sporadic AD brains [84]. Since cGMP favors the inactive state of Rab5, it is therefore possible that smaller endosomes make the approximation of APP and BACE1 easier.

Although we are still far away from elucidating the complete picture, novel molecular players governing the dynamics of Aβ production are beginning to emerge. Certainly, the more we learn on the physiology of APP and its processing, the closer we get to understanding AD and finding effective pharmacological treatments.

From this prospective, increasing cGMP levels with PDE5 inhibitors could represent a promising therapeutic strategy. As a matter of fact, a study investigating repeated administrations of udenafil in patients suffering from erectile dysfunction has shown beneficial effects on memory performance as well as on a battery of tests assessing executive functions [111].

For a more complete information, however, it must be said that, in healthy subjects, single vardenafil administration did not enhance cognition or information processing performance [112].

Chapter 5

Materials and Methods

5.1. Cell culture and treatments

Mouse Neuro-2a (N2a) cells were grown in 50% Dulbecco modified Eagle's medium (DMEM) (Euroclone S.p.A., Italy), 50% OptiMEM (Invitrogen, Carlsbad, CA) with 0.1 mM non-essential amino acids, 1% penicillin-streptomycin mixture, and 5% fetal bovine serum (FBS).

N2a cells stably expressing wild-type human APP695 (N2aAPP) were obtained from Peter Davies (Albert Einstein College of Medicine, Bronx, NY) and grown in the same culture medium with 200 μg/ml geneticin (G418).

Vardenafil (VDF), Pitstop2 and DAPT (Sigma-Aldrich, Italy) were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until use.

5.2. Total protein extraction from N2a cells

N2a cells were lysed with an ice-cold buffer containing 1 mM EGTA, 50 mM NaF, 2 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1 mM PMSF, 1% protease inhibitors (Complete Mini, Roche S.p.A., Germany) and 1% SDS. Cell lysates were spun at 15,000 x g for 10 min at 4°C and the supernatant (total protein extracts) stored at -80°C until use.

5.3. Protein quantification

The total protein concentration was determined with bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, USA). This is a colorimetric method which combines reduction of Cu2+ to Cu+ by proteins in an alkaline medium. It is based on a color change of the sample solution from green

to purple in proportion to the protein concentration. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion, making a water-soluble complex. The amount of proteins is then calculated by spectrophotometric analysis (562 nm) using a microplate reader (Bio-Rad iMark™).

5.4. Immunoblot analysis

Immunoblot analysis, also called Western blotting (WB), is a biochemical procedure that allows a semi-quantitative evaluation of specific protein expression.

In this technique, proteins are separated by electrophoresis in polyacrylamide gel [71], in the presence of SDS (SDS-PAGE). We used Bio-Rad Mini-gels system with precast gel (10 x 7.5 x 0.1 cm, 4-20 % polyacrylamide) and the standard Laemmli method [113].

Electrophoresis was performed at 140 V with a short pre-run at 70 V. At the end of the run, proteins were transferred from the gel to a polyvinylidene difluoride membrane (PVDF) using the Towbin method [114], with a cold buffer containing 25 mM Tris, 192 mM glycin and 20% methanol. Transfer of proteins from gels to PVDF membranes occurred applying a current of 100 V for 60 min. Membranes were then incubated with a solution of TBS tween (t/TBS: 200 mM Tris, 1,3 M NaCl, pH 7.5, 0,05% tween 20) and 5% milk powder, in order to saturate possible nonspecific antibody binding sites.

We used the following primary antibodies: monoclonal mouse anti-human APP (22C11, 1:1000; Millipore, Italy), monoclonal mouse anti-human transferrin

receptor (1:1000; Invitrogen, USA), monoclonal rabbit anti-Rab5 (1:1000; Abcam, UK), polyclonal rabbit anti-hemagglutinin (anti-HA) (1:1000; Sigma-Aldrich, Italy), rabbit anti-APP-CTF (1:1000; Zymed, USA) and monoclonal mouse anti-β-actin (1:10000; Sigma-Aldrich, Italy). At the end of incubations with the primary antibody, membranes were washed with t/TBS and incubated with a secondary anti-rabbit or anti-mouse antibody coupled to horseradish peroxidase (GE Healthcare, Little Chalfont, UK). Proteins were revealed with an enzyme-linked chemiluminescence detection kit (ECL, GE-Healthcare, Little Chalfont, UK). Chemiluminescence was visualized by film exposure, and signals were analyzed under non- saturating conditions with an image densitometer (Bio-Rad, Hercules, CA).

5.5. Cell surface biotinylation

N2aAPP cells were treated with 100 μM VDF for 5, 10, 30 and 60 min. Control samples (0 min) received the same volume of vehicle (DMSO) for 60 min.

At the end of treatments, N2aAPP cells were surface biotinylated by incubation with Sulfo-NHS-SS-Biotin (Pierce Biotechnology, USA) at 2 mg/ml in PBS for 30 min at 4 °C. Cells were then quenched with 100 mM glycine and lysed in 2mM EGTA, 10 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1mM PMSF, 1% protease inhibitor cocktail, and 1% SDS. After centrifugation at 15,000 x g for 10 min, supernatants were incubated for 2 h at 4°C with NeutrAvidin™ Protein immobilized onto 6% crosslinked beaded agarose (Pierce Biotechnology, USA), spun and subjected to SDS-PAGE and immunoblot analysis with the 22C11 anti-APP antibody.

5.6. Cells surface APP labeling

N2aAPP cells were grown overnight on culture slides and then incubated for 1 h at 4 °C with the anti-APP monoclonal antibody 6E10 (1:200; Covance, USA). Cells were then washed and treated with 100 μM VDF (or an equal volume of DMSO) for 10 sec or 10 min at 37°C. After fixing and permeabilization with ice-cold methanol cells were labeled with the Alexa Fluor 488 goat anti-mouse IgG (1:500; Invitrogen, USA) and analyzed with a Leica TCS SP2 confocal microscope. To confirm the specificity of the fluorescence-associated signal, we performed the labeling in permeabilized cells that were not pre- incubated with the anti-APP antibody.

5.7. Test ELISA for $A\beta_{1-42}$

$A\beta_{x-42}$ ELISA kit (Wako Chemicals GmbH, Germany) was used to measure Aβ peptides released into culture media from N2a cells. At the end of cell treatments, conditioned media were collected, spun at 1,000 x g to remove cell debris, and stored at -80°C until use. This ELISA test takes advantage of 96 well-plates coated with the monoclonal antibody BNT77, which binds to the amino acid sequence 11-28 of Aβ. Captured $A\beta_{1-42}$ is then recognized by another antibody (BC05 HRP-conjugated), that specifically detects the C-terminal portion of $A\beta_{1-42}$. The peroxidase activity is revealed after addition of 3,3',5,5'-Tetramethylbenzidine, a specific substrate of HRP. Positive samples develop a blue color. The reaction is stopped by the addition of sulfuric acid, which produces a yellow color proportional to the concentration of the antigen-antibody complex ($A\beta_{1-42}$-BC05). The absorbance is then measured at 450 nm using a

spectrophotometer (Bio-Rad iMark™). $A\beta_{1-42}$ concentrations were calculated according to the standard curves prepared on the same ELISA plates.

5.8. Plasmids and transfections

mRFP-Rab5 (Addgene plasmid # 14437) was a gift from Ari Helenius (Institute of Biochemistry, Swiss Federal Institute of Technology, Zurich) [115]; mCherry-Rab5CA (Q79L) (Addgene plasmid # 35138) and mCherry-Rab5DN (S34N) were a gift from Sergio Grinstein (Division of Cell Biology, Hospital for Sick Children, Toronto) [116]. Plasmids encoding Arf6 proteins (HA-Arf6WT, HA-Arf6Q67L and HA-Arf6T27N) were provided by Anna Fassio (University of Genoa). APP:VN (APP tagged with Venus N-terminal fragment) and BACE1:VC (BACE1 tagged with complementary Venus C-terminal fragment) plasmids were obtained from Subhojit Roy (University of California, San Diego, La Jolla). N2a cells were transiently transfected using Lipofectamine 2000 (Invitrogen, USA) at 2 μl/μg DNA.

5.9. RNA interference

Accell Mouse Rab5a siRNA – SMART pool, as well as the control Accell Non-targeting siRNA, were purchased from Dharmacon (Lafayette, USA). Transfections were performed according to the manufacturer's instructions and the efficiency of Rab5 silencing was verified by immunoblotting.

5.10. Rab5 activity

N2a cells were grown overnight on 10 cm culture dishes and then treated with 100 μM VDF for different time periods (5, 10, 30, 60 min). Control samples (0 min) received the same volume of vehicle (DMSO) for 60 min. At the end of treatments, cells were lysed and Rab5 activity was analyzed with the Rab5 Activation Assay Kit (NewEast Biosciences, USA). According to the manufacturer's instructions, cell lysates were incubated with a mouse anti-active Rab5 monoclonal antibody and the immunocomplex was then pulled down by protein A/G agarose. Levels of active Rab5 were quantified by immunoblot analysis using a rabbit anti-Rab5 polyclonal antibody.

5.11. APP-BACE1 interaction assay

To evaluate the approximation between APP and BACE1 we used the Optical Convergence of APP and BACE1 (OptiCAB) assay [107], which is based on the bimolecular fluorescence complementation of Venus protein fragments [117].

In this assay APP is tagged to the N-terminal fragment of the Venus protein (APP:VN) and BACE1 is tagged to the complementary C-terminal fragment of Venus (BACE1:VC). When APP:VN and BACE1:VC interact, Venus protein is reconstituted and fluorescent. Transfections of APP:VN and BACE1:VC expressing vectors were performed in N2a cells using Lipofectamine 2000 (2 μl/μg DNA).

5.12. Confocal analysis

N2a cells were grown overnight on culture slides and transiently transfected with mCherry-Rab5DN (S34N) where indicated. After 16 hours, cells were further transfected with APP:VN and BACE1:VC expressing vectors and, 6 hours later, incubated with DMSO or VDF (50 μM) for 16 hours. At the end of treatments, cells were permeabilized and fixed with ice-cold methanol, incubated with TO-PRO^{TM}-3 Iodide (Thermo Fisher Scientific, USA) for nuclear staining, and observed with the appropriated filters on a Leica TCS SP2 confocal microscope (planapochromat x 60 oil-immersion objective, numerical aperture 1.4).

5.12. Statistical analysis

Data were analyzed using one-away ANOVA followed by Dunnet's post-hoc. Result are expressed as mean ± standard error of the mean (SEM). The level of significance was set at $P < 0.05$.

Chapter 6

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Original Article

Investigating the amyloid-beta enhancing effect of cGMP in neuro2a cells

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ABSTRACT

Long-term potentiation (LTP) and the process of memory formation require activation of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) pathways. Notably, recent evidence indicated that both cyclic nucleotides boost the production of amyloid-beta (Aβ) peptides. In particular, cAMP was shown to favor hippocampal LTP by stimulating the synthesis of the amyloid precursor protein APP, whereas cGMP was found to enhance LTP and to improve memory by increasing Aβ levels without affecting the expression of APP. The results of the present study substantiate that cGMP has a role in the endocytic pathway of APP and suggest a scenario where the cyclic nucleotide enhances the production of Aβ by favoring the trafficking of APP from the cell cortex to the endolysosomal compartment.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by memory loss and a chronic decline of cognitive functions. The main histopathological hallmark of AD is the cerebral accumulation of insoluble amyloid-beta (Aβ), a small self-aggregating peptide generated through sequential cleavage of the amyloid precursor protein (APP) by β-secretase (BACE) and γ-secretase. The most common Aβ species contain 40 or 42 amino acids ($Aβ_{40}$ and $Aβ_{42}$, respectively) and although they have long been known to exert neurotoxic effects, a growing body of evidence supports the idea that they might play a physiological role in long-term potentiation (LTP) and memory (Puzzo et al., 2012; Ricciarelli and Fedele, 2017).

LTP is the electrophysiological correlate of synaptic plasticity (Bliss and Collingridge, 1993) and requires activation of cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA)/cAMP responsive element binding protein (CREB) and cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)/CREB pathways (Frey et al., 1993; Lu et al., 1999). Consequently, therapeutic strategies aimed at increasing cerebral levels of cAMP and cGMP are receiving much attention for their potential effect on AD and other memory-related disorders. In this context, phosphodiesterase 4 and 5 (PDE4 and PDE5) inhibitors are promising candidates, as they inactivate the enzymes that degrade cAMP and cGMP, respectively (Heckman et al., 2015a; Ricciarelli and Fedele, 2015).

The two cyclic nucleotides seem to be differentially involved in the process of memory formation. Activation of the cAMP/PKA/CREB pathway has been mainly associated with a long-lasting form of LTP (L-LTP) that requires protein synthesis and is related to the late memory consolidation process, whereas cGMP has been mostly involved in the transient early phase of LTP (E-LTP), which is independent from gene expression and protein synthesis (Frey et al., 1993; Barad et al., 1998). More recent evidence, however, suggests that either early or late LTP require both cyclic nucleotides (Bollen et al., 2014).

Intriguingly, both cAMP and cGMP have been found to boost the production of Aβ peptides. In particular, the cAMP enhancer rolipram, a specific PDE4 inhibitor, was shown to favor hippocampal LTP by stimulating the synthesis of APP and the consequent production of Aβ (Ricciarelli et al., 2014). On the other hand, more recent evidence indicated that also cGMP enhances LTP and improve memory by inducing the production of Aβ, but without affecting the expression of APP (Palmeri et al., 2017).

Here, in order to gain further mechanistic insights into the role played by cGMP in the production of Aβ, we evaluated the effect of vardenafil, a selective PDE5 inhibitor, on the amyloidogenic processing of APP.

2. Materials and methods

2.1. Cell culture and treatments

Mouse neuro2a (N2a) cells were grown in 50% Dulbecco modified

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cGMP favors the interaction between APP and BACE1 by inhibiting Rab5 GTPase activity

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Evaluating the correlation between Alzheimer's Amyloid-β peptides and glaucoma in human aqueous humor

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