Chapter 9

Physiological Role of Amyloid Beta in Neural Cells: The Cellular Trophic Activity

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Additional information is available at the end of the chapter

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1. Introduction

Amyloid is a term for the build-up of protein deposits or plaques in the body. Thus amyloid are extracellular insoluble fibrous protein aggregates. One characteristic is that these fibrils acquire β-sheet structure. Therefore the structure of the proteins that form deposits is altered and often exhibits inappropriate folding. The misfolded proteins, interact with each other and with other proteins, forming aggregates and the accumulation of these amyloid fibrils in particular organs is call amyloidosis, which is characteristic of several pathologies, including neurodegenerative diseases, such as Alzheimer's Disease (AD), transmissible spongiform encephalopathies, type II diabetes, familial amyloidoses and other variants of systemic amyloidoses [1].

2. APP processing

There are two pathways (Figure 1) for processing amyloid precursor protein (APP): An amyloidogenic pathway and a non-amyloidogenic, constitutive secretory pathway. Different APP fragments are generated after secretase cleavage.

In the non-amyloidogenic pathway, part of the extracellular domain of APP is cleaved by the α-secretases, that belong to the disintegrin and metalloproteinase (ADAM, including ADAM9,

ADM10 and ADAM17, also known as TACE), releasing a soluble extracellular fragment know as sAPP-α, that has neurotrophic and neuroprotective functions [2, 3]. Then γ-secretase [4, 5] that is present at the plasma membrane, can generate an intracellular APP fragment that is known as APP intracellular C-terminal domain (AICD) [6]. In the amyloidogenic pathway, APP is cleaved by β-secretase (BACE1) [7, 8] at its extracellular domain, giving rise to two fragments; sAPP-β (N-terminal fragment) and CT99 or CT89. Then CT99 could be cleaved by the γ-secretase complex (including Nicastrin, Anterior Pharynx defective 1, Presenilin enhancer 2, Presenilin 1 and or Presenilin 2) within the plasma membrane. These two cleavages (β-secretase and γ-secretase cleavages) generate Amyloid beta (Aβ) and more AICD fragment. The length of the AICD fragment could vary due to heterogeneous γ-secretase cleavage, and subsequent ε-secretase and ζ-secretase activity. AICD has physiological and pathological actions, particularly in signaling from the membrane to the nucleus through epigenetic modulation of gene expression [9]. Moreover inside the cell, AICD fragment can undergo more processing by caspases giving rise to a fragment called CT31, which is a potent inducer of apoptosis [10].

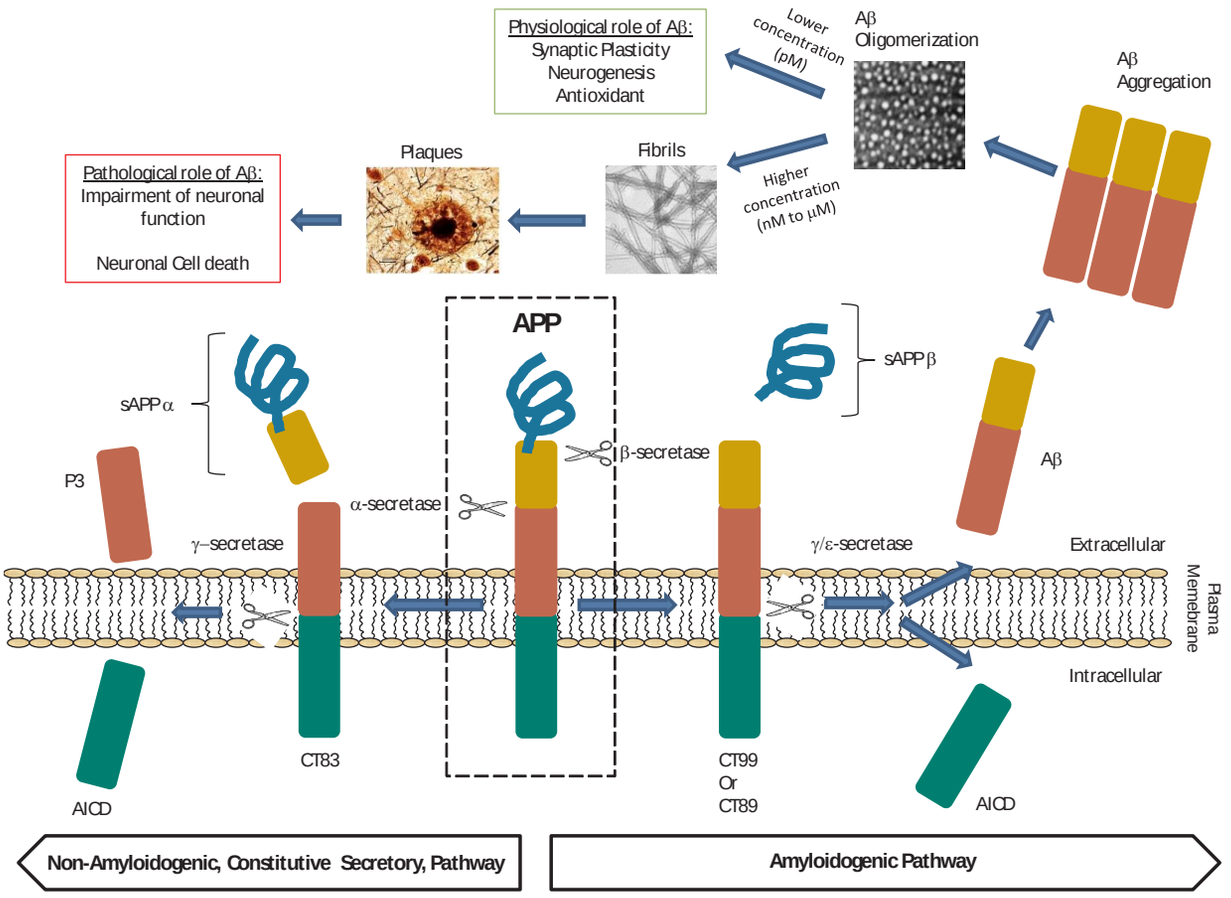

Figure 1. APP processing.

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3. Amyloid Beta

Amyloid Beta (Aβ) is a peptide generated by the amyloidogenic pathway of APP processing [11]. As we mentioned before, initially APP, a transmembrane protein, is cleaved by α- or β-secretases (Figure 1), generating large, soluble, secreted fragments (sAPPα and sAPPβ) and membrane associated carboxy-terminal fragments (CTFs). Aβ peptides could vary in size, from 38 to 43 aminoacids, being the predominant isoforms the Aβ 1-40, 90%, and the more fibrilogenic Aβ 1-42, 10% and they are generated after β-secretase (also known as BACE1, β-site APP cleaving enzyme) cleavage, followed by γ-secretase cleavage [12]. Aβ peptide has the ability of auto-aggregate, so it could exist as monomers, dimers or oligomers; which in turn can generate fibrils, that have β-sheet structure, and could deposit to form extracellular plaques (neuritic plaques) [13].

As we mentioned above, the amyloidosis is a condition in which normally soluble proteins become insoluble and are deposited in the extracellular space of various tissues. The extracellular deposits of Aβ are characteristic of several neurological conditions including: Alzheimer's Disease [14], Down's syndrome [15], brain traumatic injuries [16], and ageing [17]. Particularly Aβ is the predominant protein in the plaques, which are one of the principal histological hallmarks of Alzheimer's disease brains. Alzheimer's disease is the most common cause of dementia among older people, and is characterized by a progressive cognitive decline and loss of memory and the inability to perform common tasks.

4. Physiologycal role of Aβ

Although Aβ peptides are produced at high amounts in pathological conditions, they are also present in low levels in normal brains, particularly during synaptic activity. For many years it has not been clear the role of APP and Aβ in non-pathological conditions, and it was thought that Aβ was an incidental product of the catabolism of APP without a physiological role (Figure 2). APP is an integral membrane protein with high affinity to copper, ubiquitously expressed and it has been reported that APP is involved in neurodevelopment and is required for neuronal growth [18]. APP also participates in synaptogenesis [19] and cell adhesion. Moreover, anti-APP antibodies block memory formation [20].

At high concentrations (nanomolar to micromolar) Aβ causes neurotoxicity and cell death [21]. However, it has been proposed that low concentrations (picomolar) of Aβ could act as trophic signal [22] and as modulator of synaptic activity, with implications in memory and learning. In addition, picomolar levels of Aβ had been determined in interstitial fluid of normal brain by microdialysis [23]. According to Cirrito and colleagues, the Aβ peptide levels in the brain are dynamically and directly influenced by synaptic activity. Furthermore, low amounts of Aβ, could work as antioxidants, due to its ability to capture redox metals, such as Cu, Fe and Zn, and thus, preventing their participation in redox cycling with other ligands [24]; hence Aβ has the ability to function as a chelator and antioxidant molecule.

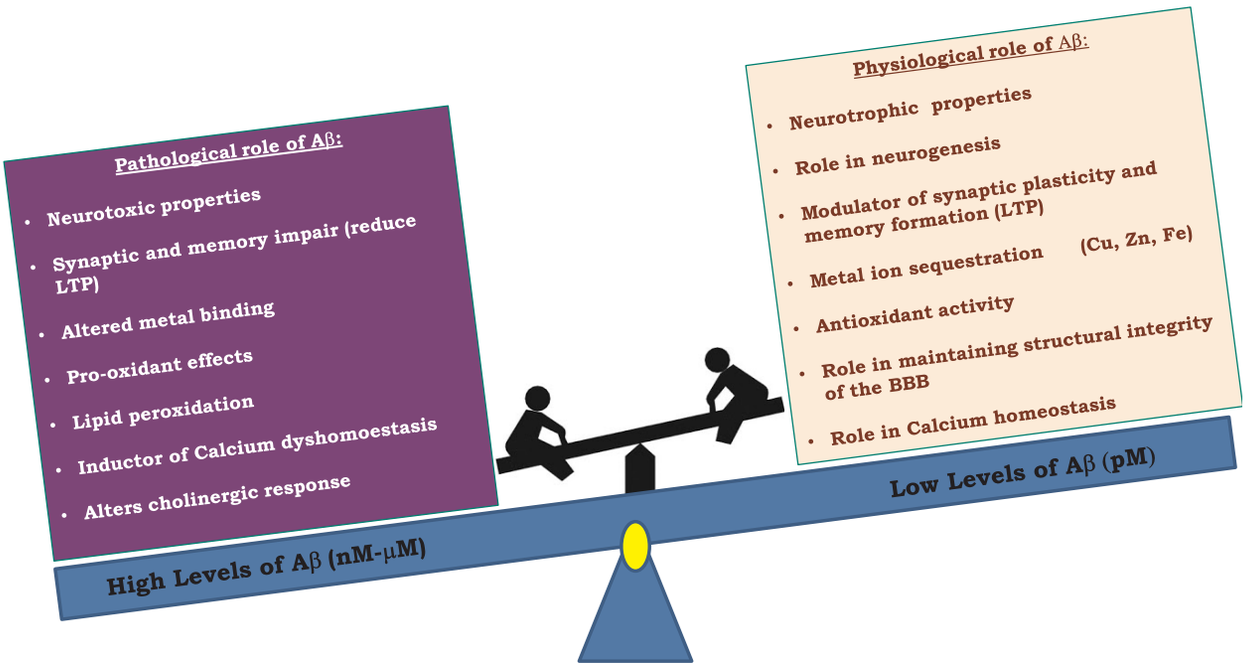

Figure 2. Balance between physiological and pathological effects of Aβ. Like a seesaw in a park, the levels of Aβ change due to environmental factors or genetic background. In normal healthy conditions, Aβ is at lower concentration (pM), and exerts its physiological functions, but in disease conditions the levels of Aβ are elevated (nM to μM) and it switches it functions to pathological effects.

Aβ has been extensively studied due to its association with neuritic plaques in AD brains [25]. However, in non-pathological conditions the existence of Aβ has also been reported. Considering it, many attempts have been addressed to find the physiological function of Aβ in the brain, particularly its role in synaptic plasticity and neuronal survival. The physiological levels of Aβ are essential for synaptic plasticity in normal individuals [26]. Taking into account the positive- or negative- effects of Aβ, it is proposed that the peptide exhibits dual effects: neurotrophic or neurotoxic. These effects may be attributed to different aspects such as its relative concentration, the cellular environment and is also related to the age of the individuals. The low physiological concentrations of Aβ could play a key role for regulating synaptic plasticity and improve cognitive functions, whereas the accumulation of high concentrations of Aβ, coupled with the effects of age, causes dysregulation and loss of synaptic function, as shown in the AD [27].

The functional properties of the Aβ have not been completely elucidated; however numerous studies have suggested that the peptide possesses neurotrophic properties [22, 28]. Recently it was suggested that soluble Aβ plays important roles in the facilitation of neuronal growth, cellular survival, in the modulation of synaptic function and defense against oxidative stress. Also, the physiological concentrations of Aβ favor the learning and memory processes [29]. In addition, it has been suggested that monomers of Aβ40, which is the most abundant species found in the brain, could function as antioxidant natural molecule by preventing the neuronal death caused by metal-induced oxidative damage.

Also, it is known that low picomolar concentrations of a preparation containing both monomers and oligomers of $A\beta_{42}$ cause a marked increase of hippocampal long-term potentiation (LTP), whereas high nanomolar concentrations lead to the well-established reduction of potentiation. The picomolar levels of $A\beta_{42}$ also produce a pronounced enhancement of both reference and contextual fear memory.

Thus, these findings here described strongly support a duality for Aβ effects in which low concentrations play a novel positive role on neurotransmission and memory, whereas high concentrations produce the well-known detrimental effect culminating in dementia [30].

5. APP and Aβ as modulators of synaptic activity

APP levels increase during synaptogenesis suggesting its role in neuronal communication [9]. There are evidences that suggest that APP interacts with the calcium sensor of synaptic vesicles possibly regulating synaptic vesicle exocytosis, and calcium homeostasis [31, 32]. The role of APP in learning and memory has been evidenced by studies showing that regulation of its levels of expression can modulate synaptic spine density, an effect that is mediated by its soluble α-cleaved fragment sAPPα [33, 34]. APP is also essential for the synapses and required for spatial learning and long-term potentiation (LTP, which correlate with memory formation) [35]. Moreover, APP participates in axonal outgrowth and restoration of neuronal functions [36, 37].

Although it is thought that Aβ impairs synaptic plasticity, it mostly depends on its concentration. High levels of Aβ have been found to markedly reduce long-term potentiation (LTP) [38], as we mentioned before, this is the type of synaptic plasticity that correlates with learning and memory, therefore, causing memory loss [25, 39, 40]. However, Aβ peptides are not only present in elevated amounts; they are also present in low levels throughout life, suggesting a possible physiological role of Aβ in normal healthy individuals [41, 42]. Recently it has been suggested that Aβ levels are likely to be regulated by synaptic activity in an endocytosis dependent manner depressing synaptic function [43, 44]. The group of Ottavio Arancio demonstrated first that low picomolar amounts of exogenous applied $A\beta_{42}$ enhance synaptic plasticity and memory [30], and second, that endogenously produced Aβ is critical for normal synaptic plasticity and memory [41]. For these latest experiments, this group used wild type mice, in which endogenous Aβ function was blocked by utilization of rodent-specific monoclonal antibody (JRF/rAb2) and by blocking the production of Aβ with the use of siRNA against APP. They concluded that endogenous Aβ is required for synaptic plasticity and memory, and that this effect is mediated via α7-nAChRs [41].

Cirrito and colleagues found that synaptic activity rapidly and dynamically regulates ISF (Interstitial fluid) Aβ levels *in vitro* in an acute brain slice model. Accordingly, the relationship between synaptic activity and extracellular Aβ levels appears to be related to synaptic vesicle release: Thus extracellular Aβ levels are increased when synaptic vesicles undergo exocytosis, even in the absence of neuronal activity. This data suggest that the rapid effects of synaptic activity on Aβ are mediated at the presynaptic side of the synaptic cleft [44].

6. Antioxidant role of Aβ

Aβ belongs to a group of proteins (metalloproteins) that capture redox metal ions (even under mildly acidotic conditions), thus preventing them from participating in redox cycling with other ligands [45, 46]. It has the ability to bind Cu, Fe and Zn [47].

Although the product of Aβ's oxidant activity is the hydrogen peroxide (H_2O_2), that is likely to mediate toxicity as the levels of oxidant rise with the increased accumulation of Aβ in the AD brain, the excessive removal of Aβ is not beneficial, since the absence of Aβ may prevent adequate chelation of metal ions and appropriate removal of O_2^- leading to an enhanced rather than a reduced neuronal oxidative stress, and this has to be taken in account when designing therapeutic strategies that use drugs that lower Aβ levels. Oxidative stress promotes Aβ generation, and in these conditions, the formation of amyloid plaques could be a compensatory response to remove reactive oxygen species [24].

One of the pathological early events that occur in the brains of AD affected individuals is the oxidative damage [48]. And also both Amyloid deposits (Plaques) and neurofribillar tangles accumulate oxidative modifications over time.

Aβ has two major sites that are important for its redox activity. The first site involves the binding of redox active Cu or Fe to human AB_{40} and AB_{42} via histidine residues that directly produce H_2O_2 by a mechanism that involves the reduction of these metal ions [49-51]. The second site is a Methionine at position 35 in the lipophilic C-terminal region [49]. The interaction of metal ions by Aβ is crucial for the redox activity and neurotoxicity of the peptide.

Several studies had evidenced the antioxidant properties of Aβ. Kontush and colleagues [52], showed Aβ prevents lipoprotein oxidation in CSF and Zou and colleagues [53], showed that monomeric $Aβ_{40}$ inhibits the reduction of Fe(III) induced by vitamin C and the generation of O_2^-. Moreover, the increased production of Aβ in mutant PS1 fibroblasts is accompanied by a decrease in the production of ROS (reactive oxygen species), particularly .OH formation [54]. Furthermore, the increased production of Aβ induced by the over-expression of wild type PS1 in brains of transgenic mice resulted in increased brain resistance to metal-induced oxidation [55]. Conversely, primary hippocampal neurons from PS1M146V mutant knock-in mice, exhibit increased superoxide production when treated with Aβ [56]. In addition to its cellular protective role, physiological concentrations of $Aβ_{40}$ and $Aβ_{42}$ have been shown to protect lipoproteins from oxidation in cerebrospinal fluid and plasma [52]. Taken together the results discussed in this section, we can conclude that Aβ can function as an antioxidant in normal neurons and many other cells, such as astrocytes, neuroblastoma cells, hepatoma cells, fibroblasts and platelets [24]. Besides, its intracellular functions, Aβ could have a metal ion binding/antioxidant role extracellularly in diffuse amyloid deposits, CSF and plasma. In this context, the release of Aβ in response to injury or disease appears to be purposive, by providing neuroprotection against oxidative stress, after which Aβ is cleared. If the clearance is insufficient (e.g. decreased neprolysin, insulin degrading enzyme or the presence of Apo e4 allele) to compensate the excessive production of Aβ, the progressive accumulation of Aβ:Cu in response to oxidative stress or in response to mutations of APP/PS1 that induce amyloido-

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genesis, may lead to the generation of H_2O_2 that exceeds the capacity of the antioxidant defense systems, further exacerbating amyloid deposition and ROS production. Thus the Aβ may not be directly toxic but the indirect generation of H_2O_2 [50] could be responsible for the oxidative damage and the neuronal dysfunction [24].

7. Aβ and neurogenesis

Current experiments in our laboratory have suggested that low concentrations of Aβ oligomers showed neurogenic effects on adult hippocampal neural stem/precursor cells (NSPCs). Currently, we are evaluating the effects of these peptides on the neuronal development *in vitro* and *in vivo* to better understand its role for the generation of new neurons under physiological conditions, based on previous work that points the trophic effect of Aβ peptides on NSPCs [57].

NSPCs are undifferentiated cells that originated from the neuroepithelium and are able to generate all cell types of the CNS (Central Nervous System): neurons and glial (hippocampus) in which neurogenesis occur during the adulthood [58]. NSPCs are of particular therapeutic interest, due to its pluripotentiality and plasticity. The idea of using NSPCs in cell therapy opens the possibility to replace damaged neuronal cells during neurodegeneration. Alternatively, the resident NSPCs in the brain could be activated and induce to differentiate through the use of growth factors, which are key regulators of the survival, proliferation and differentiation of these pluripotent cells. Trophic factors promote neuronal survival mainly through the PI3K/Akt proteins. The phospho-Akt phosphorylates and inhibits glycogen synthase kinase 3β (GSK-3β), which is one of the kinases that phosphorylates tau protein. There is evidence that suggests that this PI3K/Akt/GSK-3β signaling pathway is directly impacted by Aβ and it is altered in AD [59]. Trophic factors such as Neurotrophins (for example NGF, BDNF), IGF-1, GDNF, and hormones (insulin), are critical for neuronal survival and plasticity. Accumulations of Aβ can alter growth factor signaling and induce changes in trophic factors and its receptor (TrkA, TrkB, p75NTR, IGF-1R, Insulin receptor) expression and distribution which are characteristic of neurodegeneration [60].

a. Neurogenesis in the adult brain

Neurogenesis is a process that maintains dynamic proliferation, migration and maturation of new neurons in the adult brain and contrary to what was thought about the static nature of the brain, it has been demonstrated that the encephalon is able to generate new neurons that can be integrated into existing neural circuits. This process is finely modulated and responds to intrinsic and extrinsic factors [61-66] (Figure 3).

The formation of new neurons occurs constitutively in two well-characterized brain regions: the subventricular zone-olfactory bulb system (SVZ/OB) and the dentate gyrus (DG) of the hippocampus [65, 67-70]. However, reactive neurogenesis has also been reported in other brain regions after damage caused by harmful agents.

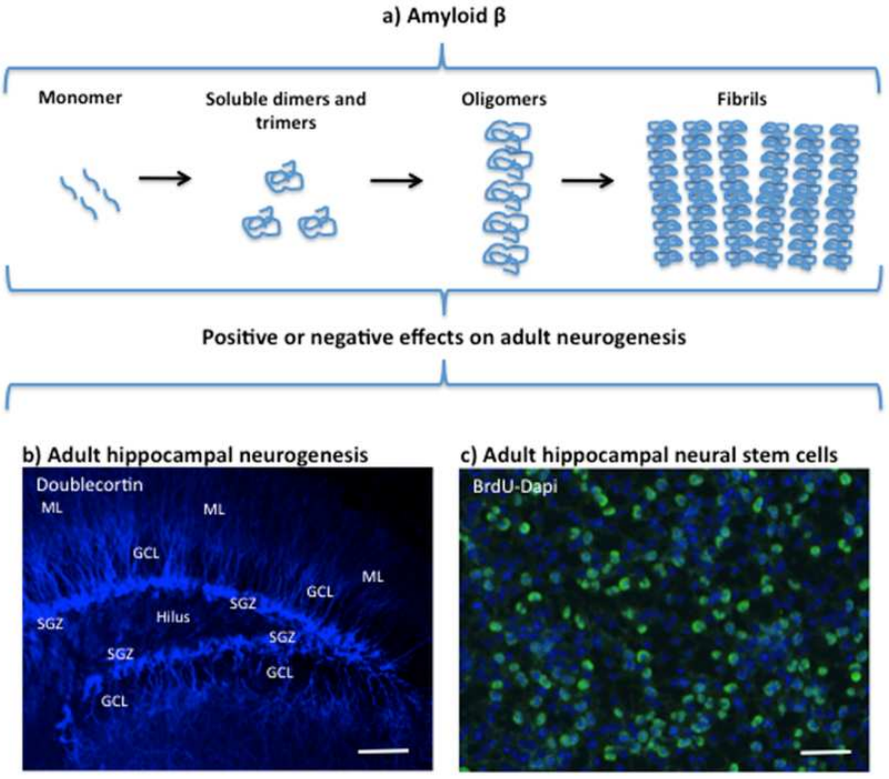

Figure 3. Role of amyloid-β in adult neurogenesis. a) Schematic cartoon indicating the different forms of Aβ (monomer, soluble dimmers and trimers, oligomers and fibrils). b) Representative picture of coronal section showing the hippocampus and stained for doublecortin (blue), a key marker for adult hippocampal neurogenesis. Doublecortin-cells line the subgranular zone (SGZ) and immature neurons migrate to the granular cell layer (GCL) to project dendrites to the molecular layer (ML). The picture also shows the hilus. Scale bar = 60 μm. c) Representative picture of proliferative adult hippocampal neural stem cells. Proliferative cells are identified by the BrdU-incorporation (green). The nuclei were stained with Dapi (blue). Scale bar = 50 μm.

Interestingly, constitutive adult neurogenic brain areas contain resident neural stem/progenitor cells (NSPCs), which have great potential for self-renewal and show multipotency [68, 71]. In the SVZ/OB system the resident stem cells located at the SVZ divide to form neuroblasts, a cellular population that migrates through the rostral migratory path to reach the OB, a place in which the immature cells become fully mature neurons [68]. A similar process occurs in the SGZ of the dentate gyrus in the hippocampus, a place in which the neural stem cells divide to form neuroblasts which will migrate a short distance into the granule cell layer to finally differentiate into hippocampal granular cells [63, 71] (Figure 3).

The fact that the stem cells of the SVZ and DG exist, makes possible their isolation to perform studies in a well-controlled cellular platform, thus many possibilities have opened to address relevant questions about the possible mechanism by which Aβ acts in a positive- or negative-manner on the neurogenic process.

b. Physiological role of Aβ in neurogenesis

Recent studies have shown that Aβ may be vital for neuronal development, plasticity, and survival due to its integral membrane interactions. Also, neuronal viability appears to be

dependent on the Aβ, a peptide that possess neurogenic properties [22]. Thus, several studies have addressed the effects of Aβ on the different events of the neurogenic process using NSPCs [57, 72, 73].

Despite the controversy about the effects of the Aβ, it is known that $A\beta_{42}$ increased the differentiation of embryonic NSPCs, an effect that was not the result of changes at the level of cell proliferation. Interestingly, this effect was only seen with soluble oligomeric forms of the $A\beta_{42}$ peptide but not with the monomeric form of $A\beta_{42}$ or with $A\beta_{40}$ or $A\beta_{25-35}$ [57]. In a similar way, but in developing neurons, Aβ induced survival and protected mature neurons against excitotoxic cell death [74]. The Aβ peptide exerts a neurotrophic role when low concentrations of the peptide are added to undifferentiated hippocampal neurons [75]. In addition, the $A\beta_{40}$ and $A\beta_{42}$ isoforms stimulate proliferation of primary neural progenitor cells isolated from rat E18 cerebral cortices [73]. Concomitant to the increase in cell proliferation, $A\beta_{40}$ induces the neuronal differentiation, whereas Aβ drives glial differentiation of neural progenitor cells into neurons [73].

In adult NSPCs derived from the SVZ, Chaejeong and collaborators [72], conducted a study with $A\beta_{42}$ peptide. In this study the three aggregated forms: monomeric, oligomeric and fibrillar, were used to evaluate their effects on the cellular proliferation and differentiation. According to the degree of aggregation or concentration of the peptides, it was found that micromolar concentrations (1 μmol/L) of the oligomeric form of $A\beta_{42}$ remarkably increase adult SVZ NSPCs. The peptide also enhances neuronal differentiation and the ability of these cells to migrate. In a similar way, recently it was reported that Aβ increases NSPCs activity in senes cence- accelerated SAMP8 mice. In the same report, but using in vitro cultures of SVZ-NSPCs, it was confirmed that Aβ promotes cell proliferation partially through a cell autonomous mechanism, in which soluble $A\beta_{42}$ exerts autocrine and paracrine effects on NSPCs.

Furthermore, the mechanisms that explain the beneficial effects of $A\beta_{42}$ have been elucidated and involved the participation of key proteins for the PI3K-Akt pathway [76]. Also, $A\beta_{42}$ acts through the p75 neurotrophin receptor to stimulate neurogenesis in the SVZ in adult mice [77]. However, it remains to be determined whether the p75 receptor is involved in neurotrophic or in the neuroprotective effects of $A\beta_{42}$. Oligomeric forms of Aβ also increase neuronal differentiation of NSPCs, acting through tyrosin kinases and MEK, but not through PI3K [57]. Although, some mechanisms have been explored, the way by which Aβ peptide targets a signal to neurogenesis remains an open question.

In addition, the physiological significance of the early increase in cell proliferation caused by Aβ is still a matter of investigation in hippocampal NSPCs models because it has been proposed that this effect causes the cessation of the new neuron formation [78]. However, it is important to consider that during aging there are also changes in the brain- and systemic- milieu, thus the decrease in the levels of neurotrophins and growth factors may also impact the neurogenic process as was previously reported [79]. Interestingly, studies performed in animal models of AD have shown that the exposure to an environmental enrichment paradigm that is capable to increase the levels of neurotrophins and growth factors, promotes the decrease in the levels of Aβ peptides and favors the neurogenic process in the hippocampus [80]. Altogether, these evidences suggest that physiological concentrations of Aβ may be relevant for promoting or

maybe to maintain adult neurogenesis. However, the direct impact of Aβ in the adult hippocampus and in hippocampal NSPCs needs to be investigated to get a full picture of Aβ roles in and during neuronal development (Figure 3).

8. Role of Aβ in maintaining the structural integrity of the blood brain barrier (BBB)

Interestingly, another Aβ trophic effect is due to its sealant properties that according to Atwood and colleagues, allows it to maintain the structural integrity of the blood brain barrier (BBB), and parenchymal structures during physiological and stress conditions [81]. In search for a therapeutic approach, the removal of Aβ (by vaccination) has been proposed, but accumulated evidence shows that low levels of Aβ had a role in maintaining the cellular homeostasis, thus complete removal of Aβ would have negative side effects. For example, Aβ could act as a sealant to maintain the integrity of the BBB, so its removal could cause leakage of serum components into the brain, resulting in an immune or autoimmune response characterized by inflammation and as a consequence it could cause also mini-strokes. In fact some clinical trials of Aβ immunization had to be halted, due to the development of encephalitis and meningitis in some patients under investigation [82].

9. Effects of high cholesterol diet on APP processing

Cholesterol is the main sterol in animal tissues, and has very important functions, as being a major component of eukaryotic membranes, and function as a biosynthetic precursor of important bioactive molecules such as steroid hormones and bile acids [83]. And also it has been shown that cholesterol can directly modulate the processing of APP [84, 85]. The main sources of cholesterol are the dietary intake and endogenous hepatic biosynthesis. Cholesterol levels and the cellular distribution of cholesterol have a major influence on amyloidogenesis [86]. The amyloidogenic processing of APP occurs in the lipid rafts (small membrane-adjacent heterogeneous domains, enriched in steroids and sphingolipids, with a role in multiple cellular processes). The β- and γ- secretases that (as mentioned before, Figure 1) participate in the amyloidogenic pathway, are located at the surface of these cholesterol-enriched regions. Accordingly, it has been reported that, increased cholesterol levels enhance β and γ- secretase activity therefore, promoting APP metabolism by the amyloidogenic pathway. Conversely a decrease in intracellular cholesterol, leads to structural rupture of the lipid rafts, favoring α-secretase non-amyloidogenic APP cleavage, leading to a significant decrease in Aβ levels [83].

Cholesterol also plays an important role in atheroesclerosis as a major component of atheroma plaques. Hypercholesterolemia is associated with the formation of atheroma plaques that progressively could cause ischemic brain damage. Brain ischemia induce an increase in APP expression, and damages the BBB [83], and as a result the clearance of cerebral Aβ is affected (Figure 4).

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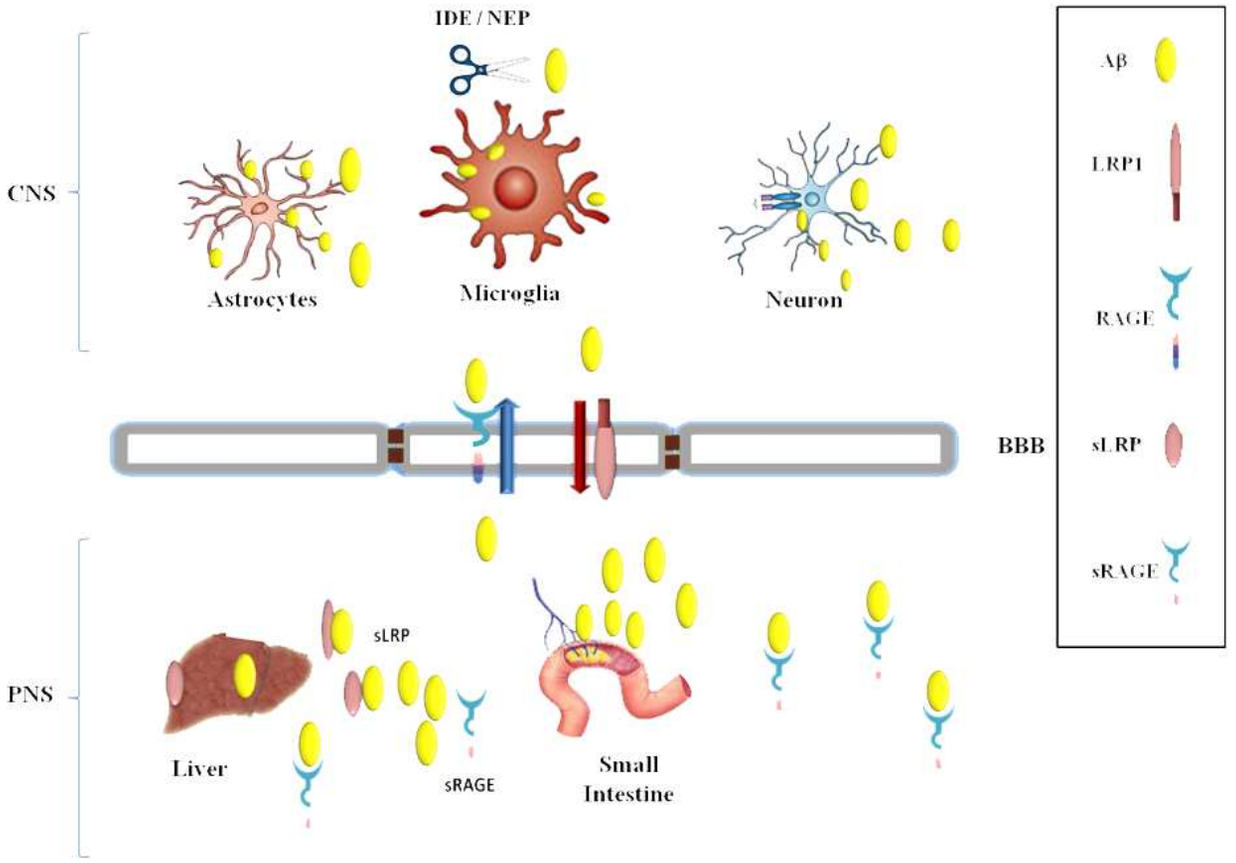

Figure 4. Clearance of Aβ peptides.A) Aβ produced by APP processing in neurons and astrocytes in the central nervous system (CNS), can be cleared by microglia phagocytosis, and further degraded by the enzymatic action of the insulin degrading enzyme (IDE) or by nephrilysin (NEP). Moreover, Aβ could be also removed by efflux through the low density lipoprotein receptor (LRP) at the blood brain barrier (BBB). **B)** Aβ peptides in the peripheral circulation could be generated at amyloidogenic organs, mainly the small intestine and the liver. In these cases, the clearing mechanisms, could involve the soluble forms of sLRP1 and the receptor of the advanced glycation end products (sRAGE). The full length form of this receptor (RAGE), is located at the BBB, and allows the influx of Aβ to the cerebral parenchyma.

The deregulation of cholesterol homeostasis and metabolism, is frequently observed in AD patients [87]. Thus it is important to consider the inappropriate diet (e.g. a diet rich in cholesterol) as a risk factor.

A set of experiments of our laboratory are focused on the evaluation of the effect of a high cholesterol diet on APP processing and generation of Aβ, based on the fact that statins (that lower cholesterol), diminish the risk of AD [83, 88]. Moreover, several reports support the possibility of a link between abnormal cholesterol metabolism and AD [86, 89-92]. According to Thirumangalakudi and colleagues, there are three principal evidences between cholesterol levels and Alzheimer disease: First, most of the genes associated with AD (that have polymorphism associated with the neurodegeneration), participate in the metabolism of cholesterol, such as ApoE, cyp46 and ABCA1. The second evidence comes from the clinical studies, which had shown that patients with high cholesterol, are more susceptible to AD [86, 93], and the third evidence comes from animal models transgenic and non-transgenic (rabbits, mice and rats), in which a high cholesterol diet have shown an enhance in brain Aβ [94-96].

Taken together, all the evidences mentioned above, it is necessary to evaluate the relationship between a high cholesterol diet and the levels of systemic and brain Aβ. We are focusing our study in the principal amyloidogenic organs (like the intestine, the liver and the brain) in rats that undertake a cholesterol enriched diet for different time periods, and evaluating if the mechanisms of clearance of Aβ are compromised and the possibility that systemic Aβ could affect or induce brain Aβ deposits possible through alterations in the permeability of the BBB. If this study shows a correlation between high cholesterol diet and elevated Aβ levels in the brain it will be tempting to speculate clinical implications directed to propose a balance diet with low cholesterol as a preventive approach for AD, as well as the use of drugs that lower cholesterol levels concomitant with the possibility of lowering Aβ levels, preferentially at early stages of the disease.

10. $A\beta_{42}$ oligomers modulate intracellular Ca2+ transients evoked by cholinergic receptors

Finally our lab is also interested in the study of the effects of Aβ oligomers on cholinergic receptors: nicotinic and muscarinic; and the role of these oligomeric forms of Aβ in intracellular calcium homeostasis. It is well known that oligomers can bind extracellular receptors [97] and indirectly activate signaling pathways. Some of these pathways could be linked to the release of intracellular calcium and the induction of cell death in cases in which the oligomeric peptide is at high concentration [98]. We are focussed on studying the consequences of Aβ interaction with the cholinergic receptors, on the levels of intracellular calcium and its impact in cell viability and synaptic transmission, based on previous reports of the role of Aβ in potentiating nicotinic receptor function and promoting oxidative stress and cellular toxicity [99].

Cholinergic pathways serve important functions in learning and memory processes. Nicotinic and muscarinic receptors are widely expressed in the brain and implicated in the pathophysiology of AD, that is the most common form of dementia, characterized by loss of neurons and synapses in the cerebral cortex and subcortical regions. The correlation of clinical dementia ratings with the reductions in a number of cortical cholinergic markers such as choline acetyltransferase, muscarinic and nicotinic acetylcholine receptor binding as well as levels of acetylcholine, suggested an association of cholinergic hypofunction with cognitive deficit, which led to the formulation of the cholinergic hypothesis of memory dysfunction in senescence and in AD [100]. As we mentioned before, Aβ is the major protein component of neuritic plaques found in AD. Evidence suggests that the physical aggregation state of Aβ directly influences neurotoxicity and specific cellular biochemical events. In addition, it has been shown that Aβ oligimers are able to modulate the release of several neurotransmitters (dopamine, γ-aminobutyric acid, aspartate, glutamate) elicited by the stimulation of cholinergic muscarinic and nicotinic receptor (mAChR, nAChR) in different brain areas. Recently it was shown the activation of both $\alpha 7$ and $\alpha 4\beta 2$ (nAChRs) as well as by the activation of mAChR modulate the Glycine release from hippocampal synaptosomes [101].

Sustained disruptions in Ca2+ signaling have significant implications for the health and functionality of neurons and form the basis of the Ca2+ hypothesis of AD [102]. Under resting conditions, cytosolic Ca2+ is maintained at low nanomolar concentrations by an array of pumps, buffers, and transport mechanisms. Ca2+ entry into the cytosol is rigorously regulated and originates from one of two major sources: the extracellular fluid via entry across the plasma membrane (through receptor-, voltage-, and store-operated channels and Ca2+ exchangers) and intracellular stores such as the endoplasmic reticulum (ER) and mitochondria [103, 104]. Interactions between Aβ and intracellular Ca2+ are particularly relevant to AD pathogenesis, as Ca2+ perturbations are a causal factor in excitotoxicity, synaptic degeneration, and cell death, whereas reduced Ca2+ release is neuroprotective [105].

In our laboratory we investigate the effects of $A\beta_{42}$ oligomers on the transient rises in [Ca2+]i evoked by cholinergic receptors in the human neuroblastoma cell line SH-SY5Y. Our results indicate that mAChR type M3 increased 56% the transient rise in [Ca2+]i evoked by carbachol in the presence of $A\beta_{42}$ oligomers, whereas the nicotine response only increased in 21%.

The experimental procedures for these set of experiments were as follows:

Briefly, preparation of oligomers was performed as reported previously by Demuro and colleagues [98]

To be able to observe the Aβ oligomers, we used atomic force microscopy. Concentrated oligomers of $A\beta_{42}$ (1 μl ~ 250 ng) were added to 9 μl double-distilled water and placed on a freshly cleaved cover slip and air-dried taken for observation by atomic force microscopy. The samples were imaged in AC-mode using a JSPM-5200 instrument (JEOL scanning probe microscope) equipped with NSC15 n-type silicon probe Al coated (μMasch), in the tapping mode. The probe has nominal spring constant of 20 to 80 N/m and driving frequencies of 265 to 410 kHz. To determine oligomer sizes we used the WinSPM system computer program provided by the manufacturer (JEOL) and Gwyddion free software for 3D analysis.

Cell culture and immunocytochemistry assays were carried out as reported before [106]. The cell line used for these experiments was the human neuroblastoma SH-SY5Y. These cells were immunostained with anti-mAChR M1 or anti-mAChR M3 (Santa Cruz Biotechnology Inc.).

For the recording experiments, the cells were seeded on recording chambers pre-coated with Poly-L-Lys. [Ca2+]i determinations in single SH-SY5Y cells were performed as reported before [107] using the Ca^{2+} indicator Fura-2AM (Molecular Probes). Aβ1-42 oligomers were applied by pipetting a fixed aliquot (50 μl) of a diluted stock solution into the recording chamber (200 μl volume). Acetylcholine, nicotine and carbachol were freshly prepared in saline solution at the indicated final concentrations. All the experiments were done at room temperature.

In the following section, we will describe our results of the experiments in which we evaluate the effects of $A\beta_{42}$ oligomers on the modulation of intracellular Ca2+ transients evoked by cholinergic receptors.

Atomic force microscopy (AFM) is used to investigate the three-dimensional structure of aggregated Aβ and characterize aggregate/fibril size, structure, and distribution. Figure 5 shows the 3D analysis of $A\beta_{42}$ oligomers morphology using AFM. The packing densities

correspond to the differential thickness of globular aggregates along a zeta axis (fiber height above the x-y imaging surface).

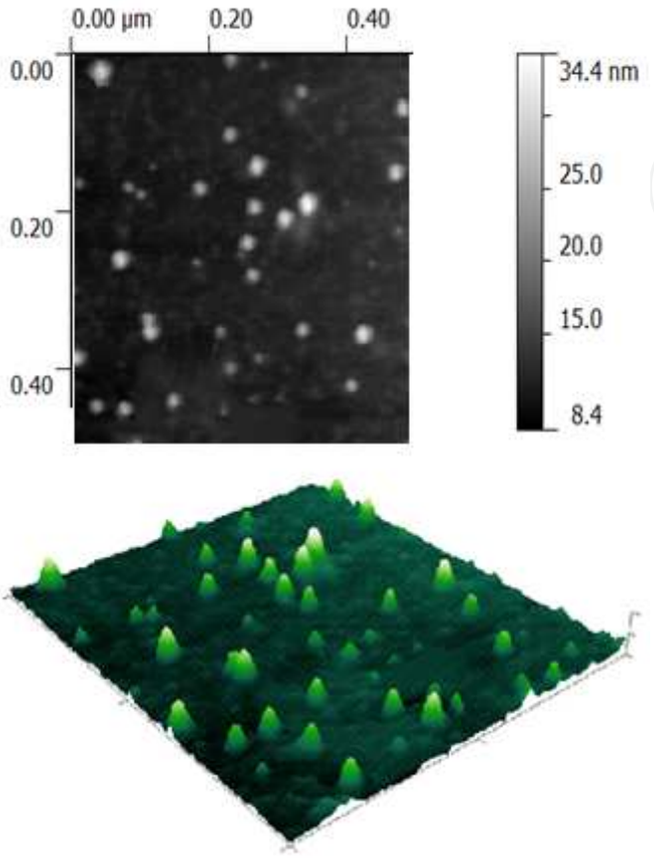

Figure 5. Aβ$_{42}$ oligomers morphology, tapping mode AFM image that shows the characteristic globular texture (scan area 540 x 540 nm).

The human neuroblastoma SH-SY5Y cells express muscarinic cholinergic receptors (mAChRs) of predominantly the M_3 subtype, which are robustly coupled to phosphoinositide (PPI) hydrolysis and Ca^{2+} homeostasis [108]. Figure 6 shows immunoreactivity for M3 and M1 receptors in SH-SY5Y cells in culture. In addition, SH-SY5Y cells express two types of nicotinic cholinergic receptors (nAChRs), ganglionic AChRs, which are normally postsynaptic and are composed of α3, α5, β2, and β4 subunits, and neuronal αBgt-binding AChRs, which are probably normally extrasynaptic composed of α7 subunits [109].

[Ca2+]i determinations in single SH-SY5Y cells:

The application of a short pulse of ACh (100 µM) to SH-SY5Y cells produced a rise in [Ca2+]i that peaked in approximately 1 s and declined toward basal levels of [Ca2+]i at the end of ACh pulse (Figure 7). When a second pulse of ACh was applied 120 s after the first pulse, the [Ca2+]i response was lightly reduced, but it was after 3 min period in resting conditions when the response recovered the whole amplitude (signaled with the arrow). Repetitive applications with 1 min interval produce progressive desensitization in the ACh response.

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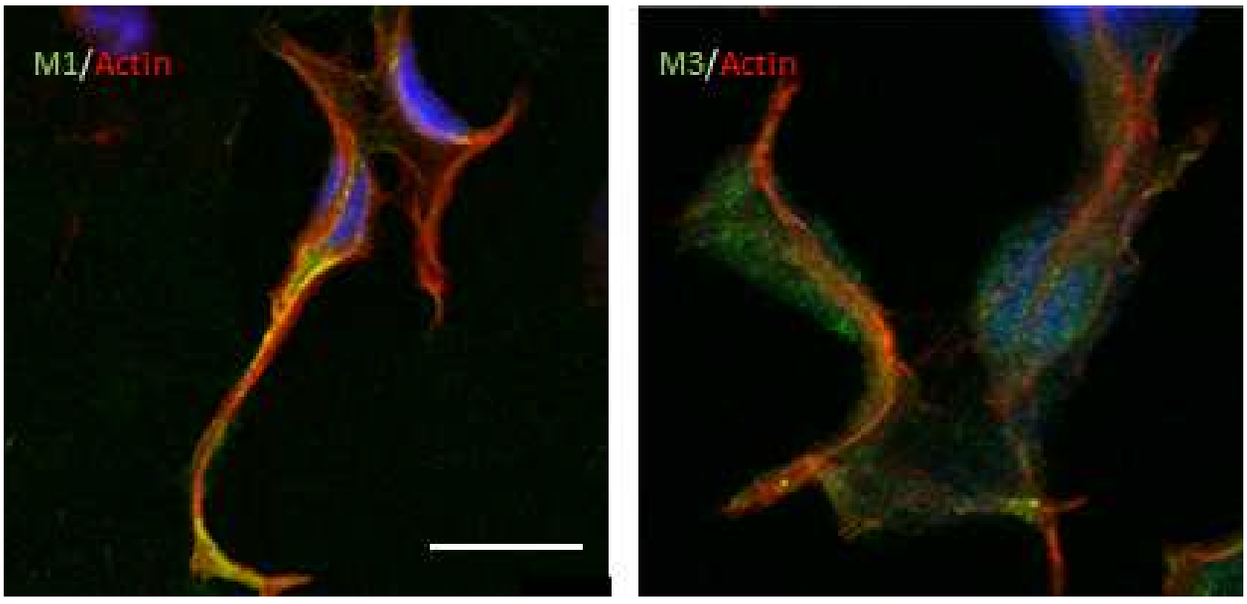

Figure 6. Confocal micoscopic localization of M3 (green staining, left panel) and M1 (green staining, right panel) immunoreactivity in SH-SY5Y neuroblastoma cells. M3 reactivity is enriched appearing finely granular and punctuate. Nuclear DNA was counterstained with Hoechst 33342 (blue staining) and the red signal corresponds to Actin inmunoreactvity. Scale bar 20 μm.

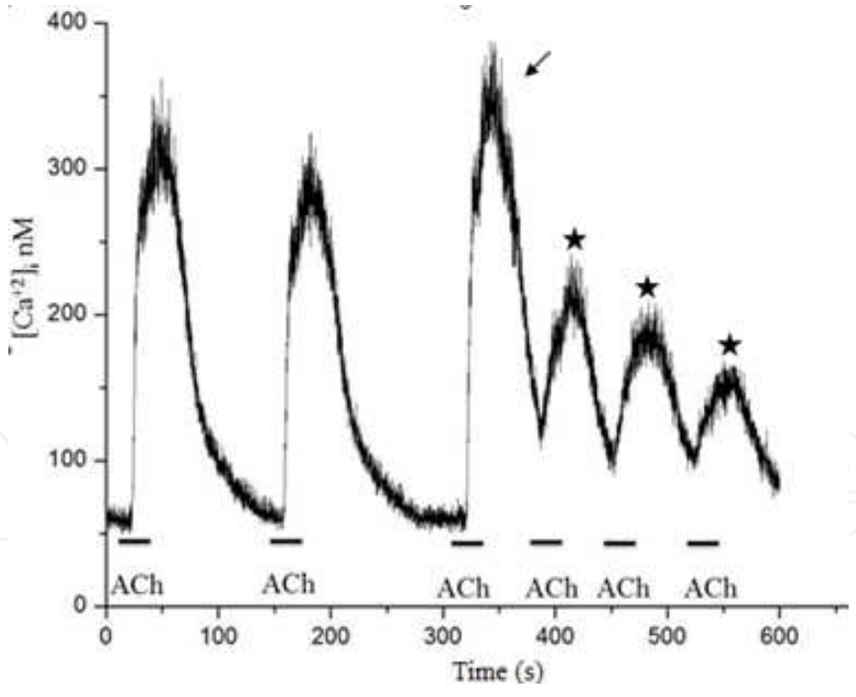

Figure 7. Transient rises in [Ca2+]i evoked by repetitive ACh pulses in SH-SY5Y cells. ACh pulses were applied during the continuous perfusion of normal saline solution (see text).

The rate of rise of the [Ca2+]i should reflect the number of activated nicotine and muscarinic receptors, however, in the most of the explored cells, the application of nicotine pulses (100 μM) was unable to produce any elevation of the [Ca2+]i, whereas in some cells the nicotinic

response was approximately 20 times smaller than those evoked by carbachol (100 μM) suggesting that the cholinergic response is mediates mainly by muscarinic receptors (Figure 8).

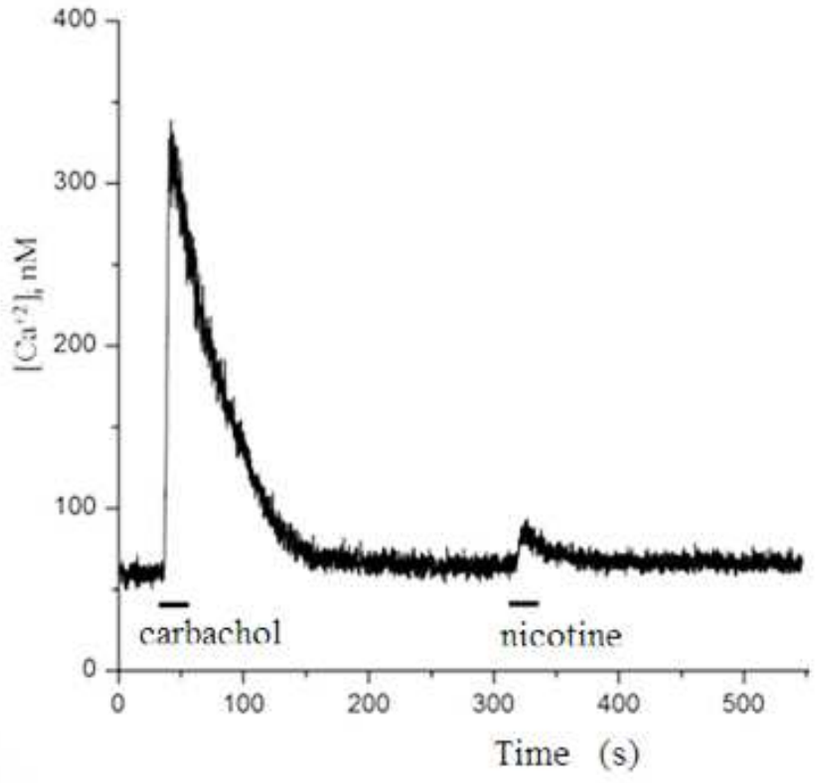

Figure 8. Transient rises in [Ca2+]i evoked by carbachol and nicotine pulses in the same SH-SY5Y cell. As in previous figure agonist pulses were applied during the continuous perfusion of normal saline solution (see text).

The incubation of SH-SY5Y cells with $A\beta_{42}$ oligomers (2.5 μg/ml) during 10 min increased 56% the transient rise in [Ca2+]i evoked by carbachol (see Figure 9), whereas the nicotine response only increased in 21%.

Taken together our results, we conclude that $A\beta_{42}$ oligomers are capable of inducing an increase in intracellular calcium levels in a dose dependent way, concomitant with an increase in intracellular Ca2+ transients evoked by cholinergic receptors. Thus the cholinergic response is potentiated by $A\beta_{42}$ oligomers. Based on previous reports (see below), our findings suggest that the increase in the transient rises of the [Ca2+]i after the incubation with the $A\beta_{42}$ oligomers evoked by carbachol, could be generated by a sustained increase of the IP3 levels, that induces a more efficient activation of IP3 receptors from the internal stores. Since ACh binding to mAChRs initiates the heterotrimeric G protein cycle, with the exchange of GTP for GDP on α-subunits and the subsequent dissociation of $\beta\gamma$ subunits, the activated, GTP-bound form of the α-subunit stimulates (or inhibits) its effector, then undergoes inactivation by intrinsic GTPase activity, which converts GTP to GDP by hydrolytic cleavage of the γ phosphate bond. Cholinergic agonist stimulation of M1, M3, and M5 receptors activates G proteins of the

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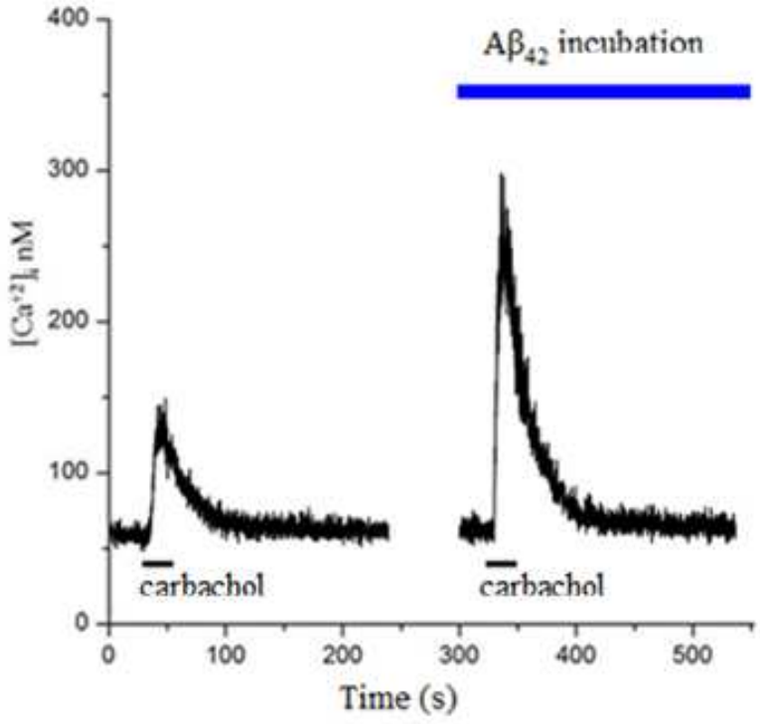

Figure 9. $A\beta_{42}$ oligomers potentiate the transient rises in [Ca2+]i evoked by carbachol in SH-SY5Y cell.

pertussis toxin-insensitive Gq/11 family. Gq/11 subunits stimulate phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate, resulting in the liberation of diacylgylcerol and inositol triphosphate (IP3). Diacylgylcerol activates protein kinase C (PKC), and IP3 induces the release of Ca2+ from endoplasmic reticulum [110]. Hence $A\beta_{42}$ induce a marked increase in the activation PKC and Ca2+/clamodulin-dependent kinase II (CaMKII) in cortical neurons, and the activation of mAChRs (M1 type) significantly inhibited the Aβ activation of PKC and CaMKII [111].

11. Conclusion

For years the Amyloid hypothesis was widely accepted as a cause of the neurodegeneration observed in AD. This hypothesis considers Aβ as a toxic factor that impairs neuronal function and leads to cell death. But recently our understanding of the physiological roles of Aβ is challenging this hypothesis.

The physiological roles of Aβ need to be taken in account in the development of therapies that intend to reduce its levels for diseases like Alzheimer's. Since excessively depleting Aβ could have negative effects, limiting its trophic functions could contribute, rather than delay the process of neurodegeneration. Furthermore, understanding the physiological functions of

APP and Aβ could help to elucidate its role during health vs disease. As we mentioned here, Aβ itself, might help to enhance synaptic plasticity and memory at appropriate concentration levels (Figure 2).

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