Analysis of the cholinergic system in Alzheimer’s disease

A study of toxicity and regulation of stress response in cholinergic cells

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To whom it may concern,

I certify that **Bertran Salvador i Mata** has carried out his T.F.G. under my supervision at the Laboratory of Physiology of the Departament de Ciències Experimentals i de la Salut de la Universitat Pompeu Fabra.

He has deeply studied the initiation of amyloid accumulation in the basal cholinergic nucleus in order to understand the onset and progression of Alzheimer’s disease. Once he has prepared the current review on the relationship between amyloid and basal cholinergic nucleus, he has designed his own plan of work that has started by the characterization of the susceptibility of cholinergic neurons to different stressors.

Along this time in our lab he has demonstrated to be a serious researcher planning scrupulous experiments as well as a good reasoner.

In summary, I think honestly that he is suitable to present the T.F.G. in our university.

Yours sincerely,

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SUMMARY

Alzheimer’s disease, the most common cause of dementia, is a chronic neurodegenerative disorder that slowly impairs the cognitive abilities of the patient, leading to a progressive neuronal loss. The main molecular neuropathological hallmarks are the intracellular neurofibrillary tangles and the extracellular accumulation of amyloid-β peptide forming senile plaques. Among the different cell types affected, early alterations have been described in the basal forebrain cholinergic neurons, together with intraneuronal accumulation of amyloid-β peptide (Aβ) on these cells, and deficits in the cholinergic system, hence hypothesizing a possible epicenter for the onset of the disease in basal forebrain cholinergic neurons, although the mechanisms and processes involved remain unknown. This project will help to better understand those mechanisms by studying basal forebrain cholinergic neurons susceptibility to stressors. The cholinergic cell line SN56.B5.G4 and primary cultures derived from the nucleus basal of Meynert of mice will be used in the study consisting in the analysis of: i) the response of cholinergic neurons to peroxide and Aβ; ii) the role of calbindin in the protection against Aβ induced neurotoxicity; iii) the intracellular trafficking of Aβ. Together with this, studies in glucose-deprivation scenarios will be performed. Moreover, slices of mice brains at different ages will be used to evaluate the trafficking of the intracellular Aβ, in order to describe possible mechanisms of Aβ delivery to other brains regions such as cortical areas or hippocampus. Altogether, this project aim’s is to provide useful information to better understand the onset and progression of the disease and to describe possible targets to prevent it.

Key words: Alzheimer’s disease (AD), amyloid-β peptide (Aβ), basal forebrain cholinergic neurons (BFCN), nucleus basalis of Meynert (NBM), primary cholinergic cultures, SN56 cell line.
THEORETICAL BACKGROUND

1.1 Alzheimer disease: molecular insights

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive impairment, being memory and language the first abilities affected. Brains from AD patients show two main pathological features: extracellular deposits of amyloid β-peptide (Aβ) known as senile plaques and intracellular neurofibrillary tangles of phosphorylated tau protein (1,2). They are also characteristic in AD the astrocytic gliosis and the neuronal death in regions such as the entorhinal cortex, the hippocampus or the amygdale (3,4). In spite of these regions, there are described affections on the cholinergic system that can occur prior to these events, and hence being important in both the onset and the progression of the disease (5).

Several hypotheses arose with the intent to explain the pathophysiological process by which the degeneration is triggered and spread for the whole brain. Evidences suggest that Aβ plays a pivotal role, as it can act as a prion and undergo brain degeneration.

The Aβ is the proteolytic product of a transmembrane protein termed amyloid precursor protein (APP) that can be processed by two enzymatic pathways: the non-amyloidogenic and the amyloidogenic pathway (Fig. 1)(6). The non-amyloidogenic is carried out by the α-secretase enzyme and prevents Aβ formation. In this pathway, two main elements are released: one ectodomin named sAPPα and a carboxyl terminal fragment C83—which could undergo further cleavage by γ-secretase (a protein complex with a catalytic center composed of presenilins) to generate different compounds important for cell maintenance-. The amyloidogenic pathway is the one that will eventually lead to Aβ formation and it is carried out by the enzyme β-site APP cleaving enzyme (BACE1) and the γ-secretase. In this case, BACE1 will cause the release of the ectodomin sAPPβ and the transmembrane fragment C99. The C99 will be cleavaged by the γ-secretase producing the Aβ. Depending on where the γ-secretase acts, different length of Aβ will be produced but mainly 40 and 42 aminoacid peptides (6,7).

Figure 1. APP cleavage. APP can undergo two different pathways: the non-amyloidogenic and the amyloidogenic. Aβ is obtained from the proteolysis of APP as a result of the amyloidogenic pathway, in which BACE1 and γ-secretase sequentially cut the APP protein.
BACE1 is a pivotal character for the amyloidogenic pathway (Fig. 1), and thus it plays an important role in AD pathology. BACE1 is a transmembrane aspartic protease that undergoes the cleavage of APP to form Aβ. In AD brains, there is an extra Aβ production as a result of an increase in the protein levels of BACE1, although most studies suggest that there is no increase in mRNA levels, thus suggesting a post-transcriptional mechanism (8,9). However, Coma M. and colleagues have proved that oxidative stress can trigger BACE1 mRNA increase (10), which is consequent with other studies that showed that other stress conditions, such as hypoxia, can as well lead to an increase of BACE1 expression (11).

Therefore, several events can lead to an increase in BACE1 activity. The major control in BACE1 expression is at the translational level. BACE1 expression is regulated by the eukaryotic initiation factor 2-alpha (eIF2-α) (9). eIF2-α, when dephosphorylated, is a protein that starts the translation process by binding the mRNA and enhance the formation of the initiation complex. However, it has been described that the phosphorylation in Ser51 of eIF2-α, which stops most of the genes translation, will act as a translation de-repressor of the BACE1 mRNA, hence increasing its protein levels. The kinases that can phosphorylate eIF2-α are PKR, PERK, GCN2 and HRI being PKR, PERK and GCN2 directly related with Aβ production in stress conditions (9,10,12). These molecular mechanisms will lead to an increase in the Aβ production, which will trigger its oligomerization as a result of a change in the peptide conformation, which will act as a prion and will aggregate in concrete regions, thus increasing the stress of these neurons, and eventually leading to cell death.

Of special interest is a possible traffic of Aβ through neuronal connections. It has been previous described the movement of APP protein through axons as a result of different stimuli, as described by Vicario-Orri and colleagues. However, Aβ is considered to be produced in situ without being affected by neuronal transport. In this regard recent studies have pointed out an intracellular accumulation in BFCN of the Aβ peptide which is not accompanied with an increase in APP expression (13) and interestingly has no pathological effects, as it is found in young healthy subjects. These observations are not in agreement with previous reports that link intracellular Aβ with an increase in tau hyperphosphorylation, and with a mitochondrial and calcium dysfunction (revised in 14). Instead, this intracellular accumulation seems to be physiological for those cells, as no toxicity is associated with its presence. Therefore, further knowledge about Aβ production and transport within BFCN, together with specific cell sensitivity will allow a better understanding of the disease. Two main questions arose from these findings: i) how it is possible that those high levels of intracellular Aβ are only found in BFCN? and ii) how can we explain that this intracellular Aβ does not cause, at least immediately, any pathological
changes in those neurons?. The answer to these questions is an important part of the project here described.

Another pivotal character in AD pathology is the accumulation of intracellular tangles composed of the microtubule-associated protein tau. Tau abnormalities have been detected in other neurological disease, called tauopathies, due to the presence of mutations in tau. Tau protein works as a regulator of the microtubule dynamics, but in AD it forms tangles, which then will correlate with the progression of the disease, as staged by Braak (4).

Tau protein has a proline-rich region together with some serine and threonine residues that produce consensus motifs for kinases, hence leading to a hyperphosphorylation, as it has been widely demonstrated in AD. Furthermore, the regions phosphorylated in AD seem to be those that are not commonly phosphorylated in normal tau function, thus suggesting that these residues become phosphorylated after tau accumulation. Moreover, this proline-rich region has an effect on tau structure and flexibility, as it allows major structural changes, a processes typically controlled by peptidyl-prolyl cis/trans isomerases, which are important chaperones in protein folding and transport. Once tau becomes accumulated and hyperphosphorylated, leading to the formation and accumulation of the intracellular tangles, there is a clear effect on cell viability and functionality, as microtubules function is comprised.

Recent studies suggest an early basal nucleus tauopathy prior to the onset of AD, as it will be discussed further on.

Therefore, both Aβ peptide and accumulation of intracellular tangles of tau protein have been early detected in basal cholinergic nucleus, thus enhancing the possibility that these neurons can play a pivotal role in the onset and progression of the disease.
1.2 Alzheimer’s disease: steps and region-specific pathology

To further understand the pathology of AD it is important to know the step-progressions of the disease together with the region-specific alterations described. The first classification of the progression of AD was proposed by Braak at 1991 (4), and it consisted of six different stages based on neurofibrillary tangles and neuropil threads distribution through brain regions. In stage I and II there are described either mild or severe alterations in the transentorhinal layer pre-α. They are followed by stage III and IV, where affections of layer pre-α in transentorhinal and entorhinal cortex, together with affection on the Ammon’s horn sector in hippocampus, are described. Finally, the isocortical stages, stage V and VI, where all the isocortical associated areas are destructed.

This stereotypical evolution of the disease could be seen approximately in Fig. 2, obtained from Eur J Nucl Med Mol Imaging (15), where a longitudinal study was performed by PET imaging to obtain the sequential impairment of different brain regions. The study was performed with different patients and by measuring cellular metabolic rate by fludeoxyglucose. This approach has some limitations, as there are no studies validating the observations with neuropathological data. However, it is useful to highlight the region-specific alterations described by Braak. Although no concrete regions such as basal forebrain cholinergic neurons or transentorhinal cortex are taken into account, it is possible to see how the first metabolic deficits occur in the hippocampus and then move to the parietal and temporal cortex to finally extend to the parietotemporal, frontal and anterior cingulated regions.

Figure 2. Longitudinal study of AD patients. (A) Four different PET analysis were performed at different ages showing the glucose metabolism reduction in different brain areas, mapped as the Z-scores corresponding to glucose metabolism reduction. B) Regional glucose metabolism values at each timepoint compared to reference control value. (IPL inferior parietal lobe, HIP hippocampus, LTL lateral temporal lobe, PCC posterior cingulate cortex, FCx frontal cortex; L left, R right hemisphere).
Although, Braak staging is considered as the stereotypical pattern by which most of the patients of AD can be classified, some cases with different progression of the disease have been described (16), being considered atypical. They can be split into two main groups: hippocampal sparing and limbic-predominant. In the case of hippocampal sparing, higher neurofibrillary tangles density was found in cortical areas, whereas in hippocampus there were lower levels. Concerning the limbic predominant atypical pathology, the density of neurofibrillary tangles in hippocampus was higher, while in cortical regions it was lower.

Therefore, at least 25% of the AD cases may not follow the stereotypical pattern of the disease. The mechanism of spread proposed by Braak, which posits that the epicenter of AD neurofibrillary tangle starts in the transentorhinal cortex, then extends to layer II of the entorhinal cortex to eventually reach the hippocampus, and from there the cortical areas, might not be the only pattern of AD progression. In fact, Murray et al (16) suggested different pattern of cell-specific vulnerability depending on the type of AD progression.

Moreover, recent research describes different regions affected by tau pathology prior to the start of Braak stage I. Therefore, tauopathy associated with AD may begin before the onset of the disease in lower brainstem(17) or in basal nucleus (5).

Mesulam et al (5) described tauopathy in cholinergic nucleus, using thioflavin-S histofluorescence and specific tau antibodies, prior to the onset of AD. Furthermore, the nucleus affected provides several cortical connections, and therefore can influence cognition and memory processes. The main outcome of the study cited was to describe the cytopathology associated with tau during cognitively normal aging, which will become worse in cognition impairment situations such as MCI or AD. These results seem consistent with the symptomatic benefits of the current treatment for AD, which consist in inhibitors of the acetylcholinesterase with the objective to increase the acetylcholine levels in brain and alleviating the cognitive impairment temporally.

Apparently, these affections on basal cholinergic nucleus happen before the onset of the disease, and might be a possible initial stage from where the pathology could spread. For that reason, further study in the basal forebrain nucleus must be addressed in order to identify a possible first stage of AD and with the intention to better detect and prevent the onset of the disease.
1.3 Basal forebrain cholinergic neurons in aging

The basal forebrain cholinergic complex is formed by the vertical and horizontal bands of Broca, the nucleus basalis of Meynert, and the medial septum, and provides cholinergic connections to the cortex and hippocampus. The neurons comprised in these regions produce acetylcholine, an organic compound that functions as a neurotransmitter and it is implicated in many roles, such as cortical development and activity, the control of cerebral blood flow, and the modulation of the sleep-wake cycle. Moreover, it has been implicated in the cognitive performance and learning and memory processes (revised in 18). Apart from the cholinergic neurons, the BFCN also contains several GABAergic interneurons connected with the cholinergic system(19). The presence of the GABAergic interneurons makes the discrimination of the cholinergic cells difficult, as there is a high proportion of GABAergic neurons. Hence, special markers such as choline acetyltransferase (13), or the nerve growth factor receptor (p75) are used to correctly identify the cholinergic neurons (20). Moreover, nerve growth factor receptor p75 is used in the sorting process of primary cultures from mice brains.

In Fig. 3 adapted from Cell journal (21), there is a scheme of acetylcholine distribution through brain. Of special interest is the red net which represents those connections between the basal forebrain cholinergic neurons with other brain regions, mainly with cortical areas, thalamus and hippocampus, together with the temporal cortex. The nucleus basalis of Meynert (nb) and the medial septum nucleus (ms) are identified in the scheme, showing its principals connections.

![Figure 3. Cholinergic system in the human brain is represented in red in the figure. The nucleus basalis of Meynert (nb) and the medial septal nucleus (ms) are connected with several cortical regions, hippocampus and amygdala, and temporal cortex, providing cholinergic innervations and playing a role in cognition and memory.](image)

Deficits in those neurons have been described during normal aging, leading to moderate loss of cognitive abilities that can be improved with inhibitors of the enzyme acetylcholinesterase, involved in the degradation of the acetylcholine. In normal aging, no cell loss has been described, but dendritic, synaptic and axonal degeneration have been
demonstrated (22–24), such as the reduction of the length of the hippocampal cholinergic fibers in aged brains, accompanied with a loss of the synaptic marker synaptophysin (24).

Several theories have risen to explain the functional impairment of those neurons in both aging and AD, such as a decrease in nerve growth factor (NGF) response, involving the retrograde transport (25,26), or the energetic deficit of those neurons, that use Acetyl-CoA not only for catabolism but for acetylcholine synthesis. Moreover, Aβ peptide has shown to decrease the levels of pyruvate dehydrogenase activities, thus possibly affecting cell viability in a glucose-deprivation scenario (27). Other hypothesis suggest that calcium might be involved, as it has been described a decrease in calbindin expression in basal forebrain cholinergic neurons, thus limiting the calcium buffering capacity of those cells and making them more prone to degeneration in the presence of stressors (28). Other proteins associated with the metabolism of calcium, such as calmodulin-dependent protein kinase I and growth-associated protein 43 were also altered in aged and AD brain, thus conferring a greater susceptibility to degeneration (29). The calcium alterations could be linked with the calcium dyshomeostasis theory of AD (revised in 30).

From all those factors a conclusion is arose: the basal forebrain cholinergic neurons are more prone to degenerate as they loss part of their capacity to adapt to stress. However, the impairment occurred in AD goes a step further, implying substantial changes in the cholinergic system.

### 1.3.1. Basal forebrain cholinergic system in Alzheimer’s disease

One of the first events described in AD concerning cholinergic neurons was the selective loss of central cholinergic neurons (31), published in Lancet in the 70s. Further research found a reduction in the number of cholinergic neurons in the nucleus basalis of Meynert, especially in Ch4 subsectors in advanced AD (32), even to the extent of 75% neuronal loss (33). The neuronal loss correlates with the severity and the stage of the disease, being specially marked in late stages. However, previous to large cholinergic loss several deficits have been described in AD brains, such as decrease in choline acetyltransferase activity and reduction of the levels of its mRNA (34). In contrast to this reduction, other transmitters as dopamine or norepinephrin do not suffer any reduction (34). Therefore, there is a specific pathology associated with AD in cholinergic neurons.

All these deficits occurring in AD seems to be restricted only to basal forebrain cholinergic neurons, not affecting other cholinergic neurons present in the brain such as the cholinergic pontine cells. Some studies suggest that the differential behavior could be explained by an increase in the metabolic activity in basal forebrain cholinergic neurons, that seems to occur in a lower extent in the brain stem (pontine cells) (35).

All these data led to the cholinergic hypothesis of AD (36), the first one proposed to explain the pathophysiological processes of the disease and the only one that led to the
development of some drugs to solve the cholinergic deficit in AD, being the only ones approved to treat mild to moderate AD to the moment (revised in 37). Of these treatments, the most used are donepezil, rivastigmine and galantamine, being donepezil the most widely prescribed. Recent studies have highlighted a possible non-cholinergic role of donepezil attenuating the Aβ-associated mitochondrial dysfunction in transgenic mice (APP/PS1) (38). However, its effect is palliative, not preventing the progression of the AD but causing a symptomatic improvement on cognition by increasing acetylcholine levels and possibly alleviating mitochondria impairment.

However, the cholinergic hypothesis of AD is still controversial, due to the fact that is hard to establish a causative relation between cholinergic alterations here described and the onset of the disease. In order to adapt the cholinergic hypothesis to current research, several new theories arose (39) with the intention to adapt new insights to the theory, as possibly the etiology of AD is a combination of several factors leading together to the development of the disease.

To further discuss the issue, better understanding of the basal forebrain cholinergic neurons is needed with the intent of relation the primary cholinergic affections described in those cells with the onset of the disease and cell loss.

Recent studies demonstrated a correlation between the loss of calbindin expression –determined by quantitative analysis using immunohistochemistry- in normal-aged brains and cholinergic cell death. Hence, two different populations of cholinergic neurons could be found in normal-aged brains: those expressing basal levels of calbindin, and those with a lower expression of calbindin. It is suggested that the cells that will undergo a degenerative process are those that lack calbindin (28). Therefore, calbindin neurons are protected from degeneration. These studies are consequent with different scenarios in other diseases, such as Parkinson’s disease, where calbindin overexpression has shown to play a protective role (40). Moreover, it has been described that the neurodegeneration occurring in the substantia nigra in Parkinson’s disease is favored by the absence of calbindin (41,42).

Another pivotal question is the understanding of cholinergic neurons susceptibility to Aβ accumulation. Different studies showed a relation between acetylcholinesterase and Aβ. It has been demonstrated that there is a co-localization of both compounds in cholinergic neurons (43). Also, there is a modulation of APP processing due to acetylcholinesterase inhibition, as demonstrated in SH cell line, where it seems to be a short-term effect on the processing of APP when acetylcholinesterase (AChE) is inhibited (44). Moreover, it has been suggested the capacity of AChE to promotes Aβ aggregation (45,46). Thus, AChE could be one of the different factors related with the differential pathogenesis in AD brains and could explain part of the cholinergic dysfunctional pattern in AD. Furthermore, soluble Aβ has demonstrated to down-regulate the production of
acetylcholine and its release (47), and several studies suggest that this inhibition on the synthesis of acetylcholine is independent to neurotoxicity, thus meaning that could be the responsible of early deficits in cholinergic neurons despite the lack of neuronal loss (48), revised in (49).

Together with this, Aβ has demonstrated an important role in an intracellular scenario, although there is a slow acceptance of the intracellular Aβ pathogenesis, mainly due to the prevalence of the extracellular theory and because of the difficulties in detecting the intraneuronal Aβ, as most of the antibodies show cross-reaction (50). Furthermore, how this Aβ is accumulated inside the cells remains unknown, as the after the cleavage of APP is released into the extracellular space.

However, recent studies were able to identify and describe Aβ accumulation in basal forebrain cholinergic neurons in a non-pathological manner, as it happens in elderly, young and AD subjects and it was region-specific, as it was only significantly detected in basal forebrain cholinergic neurons (13). This non-pathological and region-specific intracellular accumulation can help to explain the particular role of basal forebrain cholinergic neurons in the onset of the disease and the spread of the Aβ through the brain. Moreover, it has also been suggested that extracellular Aβ can up-regulate intracellular Aβ (50). In order to detect the intracellular Aβ accumulation, the protocols put special attention to avoid the use of detergents that can remove the intraneuronal Aβ peptide (50) and to choose the correct antibodies with less cross-reaction (13,51), as most of the Aβ antibodies show affinity to APP as well. Despite the technical difficulties, intraneuronal Aβ merits special attention, as some studies suggest that aggregation is more likely to happen intracellularly, as the intracellular organelles have a lower pH, higher metal ion concentrations and provide a limited space (52). Therefore, the region-specific accumulation of Aβ plus its special role in possibly enhancing aggregation highlight the importance of the cholinergic system in AD prior to the phenotypic onset of the disease. However, no conclusions can be raised yet, as there is a controversial debate concerning the techniques used, that showed different results between immuno-EM and immuno-fluorescence and light microscopy revised in (52). In spite of the difficulties, some researchers suggest the pivotal role of the intraneuronal Aβ in AD pathology (14,53).

If intracellular Aβ plays a special role in AD pathogenesis, and it has been preferentially detected in basal forebrain cholinergic neurons, possibly generated in the synapse as a result of APP axonal transport, it is possible that basal forebrain cholinergic neurons plays a pivotal role in the origin of the disease prior to clinical manifestations. How intraneuronal Aβ of the basal forebrain cholinergic neurons causes neurofibrillary degeneration and eventually cell loss requires further investigation. Understanding the reason of the accumulation, and the risk factor associated with its aggregation with age is one of the objectives of this project.
In order to further describe the relation between calbindin and Aβ in the BFCN together with other factors above described, the cell line SN56.B5.G4 resulted as a fusion of N18TG2 neuroblastoma cells with cholinergic neurons from the septal regions of 21-day-old C57B1/7 mice will be used. Different studies using this cell line has been performed to understand the pathological process occurred in cholinergic system, and different conclusions were raised. The special susceptibility of those cells to different aggressive stimuli was tested (27). In differentiated SN56.B6.G4 cells, aluminum, Aβ (at non-toxic concentrations) and sodium nitroprusside caused a higher decrease in pyruvate dehydrogenase activity than in non-differentiated ones, thus meaning that the cholinergic profile is associated to a higher sensitivity to those stressors and more prone to reach a glucose-affected scenario. Moreover, deficits in other enzymes related with the cholinergic function of those cells have been described. The conclusion of the study highlight the major susceptibility of differentiated SN56.B6.G4 cells as a result of an increase in calcium content, and the relative shortage of acetyl-CoA. Furthermore, proteomic analyses performed in SN56.B6.G4 cells after treatment with Aβ peptide revealed the up-regulation of some proteins related to stress, such as calreticulin –that binds to misfolded proteins- and the mitogen-activated protein kinase kinase 6c, while gamma-actin was found down-regulated, together with changes in the phosphorylation status of Rho GDP dissociation inhibitor (54).

By using SN56.B6.G4 and primary mice cultures of cholinergic neurons, we will give an experimental approach to the cholinergic issue, in order to find the factors that influence the specific susceptibility of the basal forebrain cholinergic neurons and with the intention to revisit the cholinergic hypothesis of AD in order to further understand the disease onset and progression and maybe to improve the cholinergic treatments used for AD therapy.
OBJECTIVES AND HYPOTHESES

From all the insights described above, three main key lines must be highlighted: i) the basal forebrain cholinergic neurons are the principle cholinergic system affected in early stages of AD in a region-specific manner, ii) the intraneuronal aggregation of Aβ in an age-independent manner seems to be restricted to the basal forebrain cholinergic neurons, and iii) the features of the basal forebrain cholinergic neurons make them prone to be affected by cytotoxic processes. Taking together, the following hypothesis is deduced:

Basal forebrain cholinergic neurons play a pivotal role in the first steps of the disease as a result of their specific susceptibility to stressors, their ability to accumulate intracellular Aβ and their axonal connections with other brain regions that will be affected in the disease in further steps. From there, several sub-hypothesis can be deduced:

I. As BFCN show higher metabolic rates (35) and different stressors decrease pyruvate dehydrogenase activity (27), BFCN can easily reach glucose metabolism-dysregulation, enhancing the phosphorylation of eIF2α (9) by the activation of GCN2 and/or PKR and PERK kinases and then leading to the production of Aβ.

II. As BFCN show early dysfunction not only in AD but in several dementia and cognitive-impairment processes, some special susceptibility of those cells to stressors might exist that make them more prone to degeneration or dysfunction.

III. As alterations in calbindin expression are shown in BFCN and those alterations are associated with degeneration (28), calbindin may play an important role in preventing cholinergic neurons impairment not only in AD but in several dementia and cognitive-impairment processes.

IV. As the loss of calbindin expression on BFCN seems to be related with age, some unknown mechanism exist that cause the downregulation of calbindin, which will be aggravated in AD conditions. Oxidative stress might play an important role on those mechanisms.

V. As BFCN do not express calbindin they show more vulnerability to neurodegeneration (40,55), therefore calbindin over-expression would be protective for cholinergic cells.
VI. As BFCN accumulates intracellular Aβ peptide (13), and in other cell types it causes toxicity (14), some unknown mechanisms may exist in BFCN that prevents its aggregation and toxicity at the very early stages of AD.

VII. As BFCN accumulates intracellular Aβ peptide (13) and they show early tau pathology (21), and intracellular Aβ has shown to induce tau hyperphosphorylation (14), and we suggest the presence of protective mechanisms in BFCN during normal aging, AD could be triggered as a result of the deregulation of those processes with BFCN as the epicenter, prior to affections in the transentorhinal cortex.

VIII. The axonal connections from the BFCN to cortical areas and hippocampus (revised in 56) could explain a possible transmission pathway, due to possible intracellular traffic of Aβ or APP.

In order to give answer to the hypothesis, four main objectives can be set.

i. To describe and characterize toxicity by using two different cell lines SN56.B5.G4 cholinergic neurons and SH-SY5Y as non-cholinergic neurons.
   i. To assess toxicity by a typical oxidative stress compound: hydrogen peroxide, at increasing concentrations. Cell viability assays will be performed in the two cell line to establish comparative analysis of stress-resistant capacities of each cell line.
   ii. To assess toxicity triggered by Aβ at increasing concentrations. Cell viability assays will be performed in the two cell line to establish comparative analysis of stress-resistant capacities of each cell line.
   iii. To assess the protective role of calbindin in presence of stressors such as Aβ or hydrogen peroxide in both cell lines.
   iv. To assess the protective role of calbindin by using inhibitors and measuring cell viability in the presence of stressors.

ii. To describe and characterize the response of the cell lines and primary cultures in the presence of stressors.
   i. Western blot analysis will be performed in order to detect the expression level of different proteins involved in the metabolism and production of Aβ (BACE1, γ-secretase, eIF2α phosphorylated and non phosphorylated, GCN2, PERK and PKR) together with other proteins such as calbindin and calreticulin, proteins involved in ER-stress responses and apoptotic proteins.
ii. Reverse transcripton PCR to assess differences in the mRNA concentrations of the proteins selected in the presence/absence of stimuli, to assess if there are changes in translation or in transcription.

iii. To describe and characterize the response of the cholinergic cell line SN56.B5.G4 and the non-cholinergic cell line SH-SY5Y to intracellular Aβ peptide.
   i. Plasmid transfection of different Aβ peptides containing differential features (different sequences including those that increase the aggregation or those that inhibit the formation of oligomers and fibrils) will be performed to assess the response of the cells to the intracellular peptide. Cell viability assays and protein levels analysis will be performed in order to establish differences between cell lines.
   ii. Plasmid transfection of calbindin protein will be performed to assess the different protective role of calbindin in different conditions: in cells treated with extracellular stressors; and in cells transfected with Aβ peptide, to assess if there is any protective role in intracellular toxicity.
   iii. Thioflavin T analysis will be performed to assess if there is intracellular aggregation in cholinergic cell lines and in non-cholinergic cell lines, to prove that in cholinergic cell lines some mechanisms will be triggered to prevent Aβ aggregation.
   iv. Western blot analysis will be performed to assess the protein levels of proteins involved in the ER stress response.

iv. To describe and characterize the expression of calbindin in cholinergic cells.
   i. Reverse transcription PCR will be used to study the transcription levels in presence/absence of stressors.
   ii. Western blot analysis will be used to study its protein levels in presence/absence of stressors.

v. To describe and characterize the transport of APP or Aβ using mice cerebral slices at different ages.
   i. Immunostaining will be performed to analyze the presence of Aβ in basal nucleus in mice brain.
   ii. Co-localization with clatrin will be performed to assess if the Aβ found in cholinergic cells is inside vesicles and therefore endocyted from the extracellular milieu.
MATERIALS AND METHODS

Cell cultures

Human neuroblastoma cells SH-SY5Y were cultured with F-12 and Glutamate medium, supplemented with penicillin and streptomycin (100 units per mL and 100 ug/mL respectively) and with FBS heat inactivated (15%).

Cholinergic hybridoma cells SN56 derived from murine septal neurones were cultured with Dulbeco’s modified Eagle’s medium (DMEM) high glucose (4.5 g/mL) supplemented with FBS (10%) and penicillin/streptomycin (100 units per mL and 100 ug/mL respectively).

Cells were incubated in a humidified atmosphere with 5% of CO₂.

Differentiation of the cholinergic neurons (SN56) was carried out by combining application of 0.001 mM all-trans retinoic acid and 1 mM dibutyryl cAMP. The differentiation was assessed by the presence of spines and further on could be confirmed by an increase in choline acetyltransferase activity.

Mouse embryo nucleus basalis of Meynert cell

Nucleus basalis of Meynert (NBM) will be isolated from 18-day-old mouse embryos, after extraction of skull and dissection of the subcortical region of interest in aseptical conditions following the protocol reported by (20). After cell culture in optimal conditions, a sorting process will be carried out to purify the low-frequency cholinergic cells found in NBM. The sorting process for murine cholinergic neurons selection takes advantage of the restricted expression of the nerve growth factor receptor (p75) in conjunction with fluorescence-activated cell sorting. Sufficient cell yield will be obtained for immunocytochemistry, reverse transcriptase PCR and acetylcholine measurements. Cultured cholinergic cells will be used after day seven, when they are supposed to be developed neurons.

Cell viability assay

MTT assay was used as a colorimetric technique for assessing cell metabolic activity, allowing the measurement of citotoxicity. Different cell types were treated with stressors in order to describe its sensitiveness. Main stressors used were hydrogen peroxide and Aβ peptide.

Cells were seeded for 24 hours in 96-well plates in serum-free DMEM medium with antibiotics and with phenol red. The cells were at a density of 10⁴ cells/ 100 microL/ well. The different treatments applied were added for a period of 22h after the seeding, with
increasing concentrations. During all the incubations time, cells were placed in the incubator (37º, 5% CO₂).

After the treatment period, medium was replaced with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent diluted in a PBS solution for 2 hours. The reaction was stopped by adding DMSO and the absorbance was measured in a spectrophotometer at 540 and 650 nm, although in some cases the parameters were adapted to include extreme values.

All the steps performed after addition of the MTT reagent were done in the dark, as it is light sensitive.

**Plasmid obtention**

Different DNA plasmids will be designed in order to transflect cell lines to identify its effect: Aβ wild-type plus a reticulum peptide signal, Aβ with a mutation at the aminoacid 18 (a synthetic non aggregant peptide) and Aβ with a mutation at aminoacid 22 (the variant Dutch, which is highly aggregogenic).

To amplify its quantity, a maxi prep will be performed. The plasmids will be defrosted and transformed in DH5α bacterial cells. DNA will be added in an eppendorf with the competent cells and they will be kept in ice for 30 minutes, after which a heat shock treatment will be applied for 45 seconds to allow the entrance of the plasmidic DNA. Once transformed, the bacterial will be grown in a LB plate with ampicilin to differentially select the bacteria that will be correctly transformed. Individual colonies will be selected to grow in liquid media overnight to amplify the amount of DNA.

A purification process will be carried out to obtain the plasmid following a commercial kit, leading to the lysate of cells and specific selection of the plasmidic DNA of interest, that eventually will be quantified using Nanodrop.

**Transfection**

Murine hybridoma SN56 cells and human neuroblastoma SH cells will be transfected with plasmidic DNA containing Aβ genes with concrete features (i.e. with RE signal or with specific mutations).

SN56 and SH cells will be seeded in 24-well plates previously coated with gelatin or poly—lisine to boost cell adhesion at a density of 8 x10⁴ per well. Cells will be grown for 24 hours with DMEM high glucose enriches with 10% FBS. For SN56 and with F12 enriched with 10% FBS for SH. Afterwards, the DNA will be transfected into each well, at different concentrations adjusted to the lipoprotein protocol. Mixed will be prepared with lipoprotein and plasmidic DNA and will be carefully added drop by drop to the cells, paying special attention to not reach the base of the well. After 4 hours, medium will be replaced and cells were incubated overnight and evaluation of the transfection will be carried out the next day.
To evaluate intracellular toxicity of the Aβ peptide after plasmid transfection, cell viability will be measured and compared to a control group.

**Protein identification by Western Blot**

Cells will be lysed with a solution containing Tris-HCl, NaCl, Nonidet P-40, dithiotreitol, protease inhibitor cocktail and sodium orthovanadate, on ice. After cell lyses, the samples will be resolved in an electrophoretic process for the adjusted time and transferred to nitrocellulose membranes using iBlot Gel Transfer System (Invitrogen). Afterwards, membranes will be blocked by Tween-20-Tris solution, containing milk and incubated overnight with the correspondent antibody to identify the proteins. Antibodies concentrations will be adjusted following the company advises: 1:500 with the anti phospho-eIF2α (Cell Signaling), 1:100 with the anti-BACE1 (Chemicon International); 1:1000 anti-eIF2α (Cell Signaling) and 1:7000 with the anti-tubulin (Sigma) as a protein control.

Primary antibodies will be diluted either with 5% skimmed milk-TTBS or 5% bovine serum albumin solution in TTBS, following commercial instructions.

Peroxidase-conjugated anti-rabbit and anti-mouse antibodies will be used as secondary that will be incubated with the membrane for at least one hour at room temperature. Afterwards, intensity of the bands will be quantified and evaluated.

**Thioflavin T spectroscopy assay**

Thioflavin T spectroscopy assay measures changes in fluorescence intensity of thioflavin T as a result of its binding to amyloid fibrils. It can be detected by both fluorescence microscopy and fluorescent spectroscopy. It is based on the benzothiazole salt, obtained from the methylation of dehydrothiotoluidine with methanol in presence of the hydrochloric acid, which is able to bind beta sheet compounds and undergo a change in fluorescence as a result of it.

It will be used to identify the aggregation rates of different cell lines, to demonstrate if there are any changes in cholinergic cell which have been demonstrated to accumulate intracellular Aβ peptide.

The protocol will be adapted to the specific cell lines and conditions defined.
PRELIMINARY RESULTS

Study of susceptibility to peroxide (H₂O₂)

Preliminary results have been obtained that describe the cholinergic susceptibility to the stressor H₂O₂. The results of the cell viability assay with increasing concentrations of H₂O₂ are shown in Fig. 4. Two different cell lines were tested: SN56.B6.G4 in Figure 4a and SH-SY5Y in Figure 4b. Results are expressed as a percentage of the controls assumed as 100%. These preliminary results suggest a major susceptibility to oxidative stressors in the case of the murine cholinergic cell line in comparison with the human dopaminergic cell line.

Figure 4. Cell viability in SN56.B6.G4 and SH-SY5Y. (A) Cell viability in SN56.B6.G4 cell line with increasing concentrations of peroxide hydrogen represented as % of the control non-treated cells. (B) cell viability in SH-SY5Y cell line with increasing concentrations of peroxide hydrogen represented as % of the control non-treated cells. Axis X represents the concentrations of peroxide hydrogen (µM).
Regarding cholinergic cells, toxicity was visualized at concentrations of 10 µM of H₂O₂, where cell viability is reduced to 15% of the total cell seeded. However, further experiments are needed to confirm this tendency.

Regarding dopaminergic cells, there showed huge levels of variation among the different conditions as seen in Fig. 4b. This behavior could be explained assuming that the concentrations from 1 to 100µM of H₂O₂ has no effect on the cells and variability could be due other factors. Therefore, toxicity is only seen at concentrations of 250 µM of H₂O₂. For that reason, more concentrations should be included between 100 µM and 250 µM of H₂O₂.

Furthermore, another cell line obtained from mice should be included, as in these results the comparison is performed between human and murine cell lines. Therefore, we cannot conclude yet that the differences observed are due to the cholinergic phenotype or as a result of the differences between human and mice cell lines.
DISCUSSION AND CONCLUSIONS

All the evidences highlighted in this project lead to the conclusion that further understanding of the role of basal forebrain cholinergic neurons in the pathology of AD could result in the finding of new potential targets to prevent the onset and progression of the disease.

Hence, if we describe how these cells response to toxicity, stressors and glucose-deprivation situations, we will be able to identify the particular characteristics of the metabolism of these cells that make them more prone to degenerate. Interestingly, this information could be useful not only for AD but for other dementias, and even for normal cognitive-impairment associated with aging, which has also been linked with dysfunction in the cholinergic system. Understanding what make these cells different, will help us to explain why there are early cholinergic deficits in AD patients, and maybe will suggest new potential targets to address efforts, as acetylcholinesterase inhibition treatment has shown to have only temporal effects.

Moreover, the existing link between basal forebrain cholinergic system and calcium buffering proteins such as calbindin or calreticulin is of great interest. It has been described that the loss of these proteins in normal aging will increase the vulnerability of the cells to any kind of stressors, as it will occur in AD. By studying calbindin and calreticulin expression we might be able to prevent neuronal dysfunction prior to the onset of the disease. It could be that oxidative stress conditions or glucose deprivation affect calbindin or calreticulin expression, hence leading to cell vulnerability.

In order to study their expression levels, it is important to take into account the concrete conditions found in BFCN: high demand of glucose, the intracellular accumulation of Aβ peptide, the deregulation of pyruvate dehydrogenase induced by Aβ, early expression of hyperphosphorylated tau protein, or the phosphorylation of eIF2α. Therefore, if we are able to link the differential expression of calbindin and calreticulin in BFCN with some of the factors described, we might be able to understand which conditions are especially dangerous for AD onset.

Related with the intracellular Aβ peptide shown in BFCN in young individuals, some information could be obtained. As this Aβ seems to not be pathological for BFCN cells, it must exists some protective mechanism that allows a proper management of it, as it has been described as toxic in other cell types when accumulated intracellular. Concretely, it has shown to increase the levels of tau hyperphosphorylated protein and to cause mitochondrial dysfunctions. Therefore, understanding how this Aβ is accumulated and how it is related to tau protein might help to better prevents the pathological events that trigger the disease.
In parallel with the study of BFCN susceptibility and toxicity, the study of the possible Aβ or APP traffic through axons from BFCN to cortical regions and hippocampus will help to understand the mechanisms that allow the progression of the disease. It could be that the Aβ that is produced in a large extend in the BFCN is controlled by unknown mechanisms, but at some point could move to other neurons as a result of a pathological impairment of the Aβ control. If intracellular Aβ is able to move between neurons, in pathologic or non-pathologic scenarios, will enlighten a possible target to prevent the progression of the disease. Therefore, the study of Aβ traffic is of great interest for the prevention of the disease progression.

Altogether, the possible knowledge that can be derived from this project will enlighten several important processes involved in disease onset and progression that currently remain unknown.
REFERENCES


