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ABNORMAL NEOCORTICAL EXCITABILITY AS A MECHANISM UNDERLYING EARLY COGNITIVE DEFICITS IN ALZHEIMER'S DISEASE

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1. ALZHEIMER'S DISEASE

1.1 ALZHEIMER'S DISEASE

Neurodegenerative diseases are chronic, progressive, and age-related conditions, which cause a selective death of neurons in specific brain regions finally resulting in dementia. The condition of dementia consists in a gradual cognitive decline which significantly affects the daily activities of the concerned subjects and is one of the primary causes of reduced autonomy, disability, and death among the elderly population.

Alzheimer's disease (AD) is the most common neurodegenerative disease.

On a neuropsychiatric level the most prevalent symptoms of AD is the progressive decrease in episodic memory in elderly individuals, followed by cognitive, language, and orientation impairments, eventually resulting in a decline in executive and vegetative functions, finally leading to a complete loss of independence correlated with a decline in the patient's quality of life. From an histological viewpoint, the primary characteristic components of AD are the accumulation of i) β-amyloid peptide (Aβ) and consequently amyloid plaques within the brain and ii) hyperphosphorylated and cleaved forms of the microtubule-associated protein tau, with the generation of neurofibrillary tangles. Whereas on a neuronal level, severe changes in the neurons' activity can be detected in AD (Muñoz et al., 2019).

Regardless of the crucial scientific advancements in the clinical-diagnostic classification of this syndrome, since Alois Alzheimer described the first clinical case in 1907, we still lack a complete understanding of its aetiology and pathogenesis, making it hard to identify effective treatment options and, consequently, efficiently apply them to interrupt the progress of the disease (Oboudiyat et al., 2013).

1.1.1. Socio-economical role

An increase in the world's population and its average age has led to a corresponding rise in the incidence rate of these conditions, making neurodegenerative diseases, and specifically Alzheimer's disease, acknowledged by the World Health Organization as a priority for public health. Currently, this condition affects 44 million individuals globally, but estimates predict that this number will triple within the next 30 years.

In industrialised countries, the percentage of affected individuals is approximately 8% of the population over 65 years old and over 20% of the population over 80 years old. Furthermore, Alzheimer's disease represents the most prevalent type of dementia, accounting for 50-75% of all cases, and primarily affecting the elderly population (Lane et al.,2017).

1.2. DEVELOPMENT OF THE DISEASE

The onset of Alzheimer's disease is slow and gradual. Its symptoms present with a variety of nuanced features, particularly in the initial stages, which often results in delayed diagnosis. They are usually made following a request from the patient's family members or caregivers, who witness a decline in the cognitive functions and encourage the individual to undergo further check-ups and assessments.

The most symptomatic and noticeable characteristic of AD is the loss of memory. At first, the patient presents gradual impairment in working memory, manifesting higher sensitivity to distractions, and affecting problem solving and goal-directed behaviour (Holger et al., 2013). Consequently, cognitive deficits extend to episodic memory, the recall of life events, eventually resulting in the gradual loss of declarative memory, followed by deficits in the non-declarative one.

In the following stage, one of the functions that is severely affected is language regulation resulting in aphasia, specifically, naming difficulties known as anomia. Nevertheless, subjects often learn to adopt circumlocutions to compensate, making the deficit go easily unnoticed. Afterward, the decline expands to the formulation of speech, verbal fluency, and semantic categorisation.

Moreover, significant psychological and behavioural conditions such as anxiety, depression, delusional ideas, and hallucinations present themselves as comorbidities of this severe pathological condition. This results in irritability, aggressiveness, apathy, repetitive or purposeless behaviour, insomnia, reduced appetite, and a tendency to eat food out of schedule or without physical hunger (Bondi et al., 2017).

Dementia can present itself in a prodromal form called Mild Cognitive Impairment (MCI) which is considered an intermediate phase between a healthy aging and a pathological condition. This phenomenon can either proceed to turn into dementia, stabilize or even improve depending on its cause, subtype, and the treatment in action (Sanford A. M., 2017).

1.2.1 Mild Cognitive Impairment

The concept of mild cognitive impairment (MCI) was introduced in 1999 by Ron Petersen, Glenn Smith and colleagues to describe a condition characterised by an impairment of cognitive functions, particularly memory, that is more severe than would be expected with

advancing age, but not severe enough to be considered dementia; it occurs in a percentage ranging from 3% to 22% of the over-65 population (Sanford A. M., 2017).

There are numerous risk factors that play an important role in the development of MCI, including age, followed by male sex, the presence of the ε4 allele of alipoprotein E, a family history of cognitive impairment, the presence of several vascular risk factors, and a sedentary lifestyle (Roberts R. & Knopman D.S., 2013).

However, numerous studies have shown that MCI regresses to a state of cognitive normality in a segment of the population ranging from 30% to 50%, particularly when the effect of MCI is limited to impairment of a single cognitive domain, in a general situation characterised by the absence of the ε4 allele of alipoprotein E, better hippocampal volume and better results in cognitive tests.

Despite the encouraging data, some studies have shown that between 5% and 10% of people with MCI later progress to dementia each year, showing a much higher rate compared to 1% to 2% of the population with no history of cognitive impairment (Sanford A. M., 2017).

There are five clinical criteria used to diagnose MCI: (1) a significant change in the subject's cognitive capacity compared with a reference population of the same age and education; these changes must be confirmed by family members or declared by the patient himself. (2) Presence of disturbances in one or more cognitive areas (memory, executive functions, language, attention and visuospatial abilities), demonstrated by specific tests. (3) Episodic memory deficits in individuals with a tendency to progress to Alzheimer's disease. (4) Preservation of autonomy in activities of daily living, although subjects may take longer or perform less efficiently. (5) The subject must not be in a state of dementia.

Mild cognitive impairment is diagnosed using neuropsychological tests and various neuroimaging techniques, and in the specific case of the pre-Alzheimer's subtype, is manifested by amyloid plaques in the brain, which can be visualised by positron emission tomography (PET), and a reduced hippocampal volume, which can be detected by Magnetic resonance imaging (MRI) (Roberts R. & Knopman D.S., 2013).

Several types and subtypes of MCI have been identified. The first distinction is done between amnestic and non-amnestic type, further subclassified in monodomain or multi-domain. The ratio between amnestic and non-amnestic MCI is believed to be around 2:1. The amnestic monodomain subtype of MCI is considered the main prodromal form of AD,

presenting a cognitive profile characterized by memory deficits (Tangalos E. G. & Petersen R. C., (2018).

1.2.2. Stages of the disease

As previously stated, the condition of MCI might evolve into dementia, specifically Alzheimer's disease, which progression follows four main phases.

Reactive or initial phase

The first symptoms appearing in this disorder involve memory, specifically, episodic memory, and spatial and temporal orientation. The affected individual begins to realise that they can no longer perform complex tasks independently. This may lead to depressive and/or anxious reactions, compensatory or denial behaviour, and avoidance.

The patient retains a typical level of autonomy and self-sufficiency during this initial phase.

Neuropsychological or Moderate phase.

At this stage symptoms from the first phase intensify, leading to challenges in carrying out fundamental everyday activities. The decline in executive and cognitive abilities becomes more incapacitating, and the individual often loses independence, needing a considerable support.

Neurological or moderate-severe phase

In this phase, there is a severe impairment of cognitive and executive functions, with critically impaired memory, making daily life challenging both for the patient and their caregivers. At this point symptoms of psychological and behavioural dementia such as repetitive behaviour, hallucinations, delusions, apathy, and even incontinence manifest themselves.

At this point, the individual is entirely dependent and requires consistent support.

Internistic or severe phase

At this stage of the disease, the patient loses complete control over his cognitive functions. Vegetative functions are severely impaired, and the patient experiences incontinence and inability to walk or eat independently. Thus, the subject is compelled to endure a state of necessary bed rest, which leads to further deterioration in their health, causing bedsores, sensory and cognitive deprivation.

Finally, it is recommended to hospitalise the patient in a well-equipped healthcare facility (Làdavas E. & Berti A., 2014).

1.3. BIOLOGICAL MARKERS

Dementia is commonly caused by neurodegeneration, a condition that involves the gradual loss of neurons' typical structure or function, ultimately leading to either their death, or macroscopic structural changes of regions in the brain. There are several types of dementia, and some of them are caused by specific mechanisms that cannot always be defined in vivo. Alzheimer's disease, in particular, can only be confirmed through the post-mortem analysis of the brain tissue (Bondi et al., 2017).

1.3.1. Genetic factors

Although the major cause of Alzheimer's disease is unclear, there is a rare autosomal dominant form called familial or early-onset AD (fAD).

fAD is caused by a mutation in one of three genes: Amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2). The mutations in these genes lead to an increase in the production of β-amyloid peptide, which is responsible for the formation of senile/amyloid plaques accountable for the disease. In the case of this rare fAD, symptoms usually show up between the age of thirty and fifty (Lane et al., 2017).

There are over 20 genetic factors that increase the risk of developing the most frequent form of AD– late-onset AD. Notably, the apolipoprotein E gene, in particular, the ε4 allele is the most significant risk factor.

The apolipoprotein E gene can have three alleles: ε2, ε3, and ε4. The ε4 allele contributes to the buildup of extracellular amyloid plaques, and intracellular neurofibrillary tangles, the defining characteristics of the disease that result in a malfunction in protein synthesis and degradation, causing dysfunction, atrophy or hypoactivation of certain brain areas, as well as other subcortical structures.

Moreover, the manifestation of this condition is often linked to changes in neurons' excitability, rhythm, and signalling, which particular protein combinations, including ion channels, regulate (Chang et al., 2019).

1.3.2. Biomarkers

As mentioned above, the main biomarkers of AD can be identified as abnormal extracellular accumulation of Aβ and intracellular neurofibrillary tangles (NFT).
In early stages of AD the origin of memory deficits has been found in the combined effect of soluble Aβ and tau at synaptic level, while in more advanced phases of the disease, Aβ and NFT structurally damage the brain causing cortical degeneration and synaptic loss. Moreover, evidence shows that injections of tau and oligomeric Aβ in animal models lead to memory impairments and damaged synaptic plasticity thus confirming their role in the development of the disease (Sehar et a., 2022; Nicole O., 2016).

Evidence indicates that, besides the accumulation of Aβ, amyloidosis-induced tau hyperphosphorylation and microglia activation have been found to alter dendritic spines both functionally and structurally, contributing to spine loss in the system.
NFTs originate from an abnormal hyperphosphorylation of tau protein. In healthy conditions tau protein is involved in the polymerization of tubulin into microtubules and their stabilization, promoting axonal functionality. By contrast in AD tau protein becomes insoluble and eventually results in pathological intracellular accumulations in the form of filamentous structures.
Microglia, on the other hand, is generally involved in active immune defence thanks to its neurotrophic properties, nonetheless in the AD brain it can play a neurotoxic role producing several pro-inflammatory mediators and potentially neurotoxic substances, further contributing to CNS injury. Therefore, pathological microglia activation leads to a condition of neuroinflammation, characterized by clusters of activated microglia and astrocytes around amyloid plaques (Stojić-Vukanić et al., 2020).

Moreover, Aβ peptide is derived from APP, it aggregates extracellularly in the form of insoluble densely packed filaments-plaques, and heavily contributes to the condition of neurotoxicity in the brain (Hadzibegovic S., 2015) damaging cellular respiration, energy production, and mitochondrial activities, leading to synaptic damage, neuroinflammation, and oxidative stress.
It is believed that Aβ accumulation occurs prior to tau pathology; however, the relationship between these phenomena is not clear yet.

The amyloid cascade hypothesis, which posits that the deposition of the Aβ peptide in the brain is a central event for the development of AD, is currently widely accepted and studied (Sehar et a., 2022).

1.3.3. Amyloidopathy

APP is a gene located on chromosome 21 (Sehar et a., 2022) which is important for neuron generation, growth, differentiation and migration, as well as playing a protective (Hadzibegovic S., 2015) role in long term potentiation by strengthening synapses to enhance signal transmission through regulation of calcium release (Sehar et a., 2022).
APP can be processed through various sets of enzymes, resulting in two pathways – the amyloidogenic one, leading to the production of Aβ, and the non-amyloidogenic one. Generally, the non-amyloidogenic pathway involves around 90% of APP, with the amyloidogenic one accounting for the remaining 10%. However, these proportions can be altered by genetic or environmental factors, as well as by the ageing process.
Most AD mice are based on transgenic overexpression of APP in combination with various familial AD-associated mutations in the APP or PS1 genes. This overexpression of APP creates heightened Aβ levels to resemble the Aβ amyloidosis of AD brains, but also results in increased levels of APP fragments automatically (Hadzibegovic S., 2015).

Aβ deposition in the brain might have a functional effect in picomolar concentrations. It is believed to modulate synaptic plasticity, neurogenesis and long-term potentiation. It also has neuroprotective and antioxidant properties, reducing apoptotic death in nanomolar concentrations. However, Aβ-induced neurotoxicity may lead to synaptic dysfunction, excitotoxicity, changes in membrane permeability, inflammation, alteration of calcium homeostasis, and oxidative stress.

In healthy conditions, Aβ is cleaved by β -secretase enzymes, which degrade it into soluble APP fragments. These fragments are further cleaved by γ-secretase enzymes creating peptides that are released outside the cell and quickly removed.
By contrast, in AD pathology a dysregulation in the homeostasis of the secretase occurs resulting in the production of insoluble Aβ peptides (Sehar et a., 2022). Aβ1-42, especially, is regarded as the harmful peptide due to its increased propensity for misfolding and

accumulation in the form of Aβ plaques, which lead to cell degeneration and consequent neurotoxicity.
Furthermore, Aβ oligomers can alter their structure to reflect the action of ion-channels on the cell membrane, leading to a pathological intracellular accumulation of calcium that contributes to synaptic toxicity. This condition of Aβ-induced neurotoxicity in the brain will be further addressed in the chapter below.
Aβ concentration is based on the equilibrium between its production and clearance rate in the brain, provided by the influx/efflux dynamic of this peptide across the blood-brain barrier (BBB) alongside the process of enzymatic degradation.
Aβ production rate is thought to be at 7.6% per hour and a clearance rate of 8.3% resulting in a greater Aβ clearance than production, thus limiting its accumulation. However, the rate of clearance of Aβ1-42 in AD was found at a level of 5.3% per hour, leading to its pathological depositions.

At first, it was believed that the origin of AD- induced cognitive impairment was to be found in the formation of Aβ fibril deposition in amyloid plaques, however, cognitive impairments in mice models of AD are detected even prior to the appearance of the plaques confirmed by a higher correlation between memory deficits and Aβ deposition rather than plaques number (Hadzibegovic S., 2015).

1.4. TREATMENT OPTIONS

Alzheimer's disease is one of the most important and prevalent neurodegenerative diseases affecting the elderly. One of the major challenges associated with AD is its late diagnosis. This is generally due to the fact that early cognitive deficits are easily overlooked by patients and their families, leading to diagnosis at an advanced stage of the disease. By this time, the pathological processes have irreversibly damaged the brain, so finding an early diagnostic tool and intervening at an early stage of the disease is a very important goal for Alzheimer's research.

Since Aβ is detectable at very early stages of the disease, appearing before the onset of clinical symptoms, increasing Aβ levels in the brain are considered an effective candidate for an early biomarker of AD, which could be useful for early diagnosis and subsequent application of an early therapeutic approach.

Currently, there is no cure nor treatment available that can stop the progression of AD.

The most effective approach is to personalize the treatment plan according to the patient's condition and the stage of the disease. It is crucial to formulate any rehabilitation intervention based on the severity of the pathology in the individuals concerned.

In subjects at an early stage of the syndrome, interventions are often administered to stimulate specific cognitive functions that have not yet deteriorated, aiming to limit and delay their impairment. In contrast, patients in more advanced stages are treated with approaches focused on the care, recreation, and containment of more severe behavioural symptoms. During the most advanced stages of the disease, it is crucial to implement a multidisciplinary intervention involving specialists from the fields of medicine, psychology and social support services.

Viable treatments comprise memory stimulation, reality orientation interventions, behavioural and occupational therapies, music therapy, cognitive activation training, and pharmacological treatments (Bisiacchi & Tressoldi., 2009).

2. INTRINSIC EXCITABILITY

2.1 NEURONAL PLASTICITY

In the latest years, a relevant question the neuroscientific community has been trying to answer revolves around the topic of how neurons within our brain manage to encode and consolidate new memories.

The process responsible for this phenomenon can be found in the mechanism of neural plasticity, which is a property typical of the cerebral cortex that allows neural connections in the brain to undergo a series of functional and/or structural adapting alterations affecting the neuronal excitability following a certain experience or event (Baroncelli & Maffei, 2010).

The term neuronal excitability is used to describe the input/output dynamic behind the mechanism that allows neurons in the central nervous system (CNS) to convert inhibitory and excitatory inputs into a particular output, named action potential (AP), consequently propagated along the axon of the cell to the following one. Said signal is determined by the combination of both synaptic connectivity features, namely synaptic plasticity, and intrinsic membrane properties such as the number, distribution, and activity of a variety of membrane ion channels and receptors as well as the balance of intracellular and extracellular ion concentration.

Synaptic plasticity, precisely, refers to the mechanisms occurring at the synaptic level, which, depending on the experience modify, strengthen, or weaken the efficiency with which electrical signals are transmitted from one neuron to another. An example of these processes is the alterations of the efficiency of excitatory and inhibitory connections respectively classified as long-term potentiation (LTP) and long-term depression (LTD). These phenomena have become of great interest following the discovery of their vital role in memory encoding and consolidation, and their capacity to be induced by behavioural training.

In addition to synaptic plasticity processes, intrinsic membrane properties also play a crucial role in actively influencing the regulation of neuronal excitability (Wijesinghe & Camp, 2011).

'intrinsic excitability is one example of a learning-related change in neuronal plasticity and reflects alterations in the way a neuron responds to incoming information (e.g. from a learning event or synaptic stimulation) […] is often learning-specific (i.e. it does not occur in

animals that do not learn), transient (i.e. it lasts for a brief period of time after the learning event), and can be observed in specific subpopulations of neurons that likely reflect the memory trace. Thus, intrinsic plasticity is thought to be a substrate of learning that is independent of synaptic changes' (Yousuf et al., 2020).

Intrinsic excitability shapes the processes involved in information transmission, precisely presynaptic release of neurotransmitter, postsynaptic transduction, synaptic integration, action potential (AP) output, back-flow of information into the dendritic arbors, and retrograde signaling towards the presynapse (Frick & Johnston, 2005).

One of the factors that plays a key role in the intrinsic excitability of the cell is the membrane potential, which determines the amount of excitatory current necessary to reach the action potential threshold (Dunn et al., 2019). Said value is influenced by the permeability of neurons to different ions, achieved by modifying its voltage-gated ion channels (Yousuf et al., 2020). This is considered a very relevant point since previous research has shown that the resting membrane potential (RMP) is often found altered in age-related conditions and disease states (Dunn et al., 2019) and the regulation of voltage-gated ion channels' density is suspected to be potentially associated with cases of central nervous system (CNS) dysfunction (Yousuf et al., 2020). The neuronal membrane presents a variety of potassium-selective ion channels, which include voltage-gated and calcium-dependent channels, both vital for the regulation of membrane excitability. The activation of these channels act on the membrane potential by shifting its value towards the potassium equilibrium potential, thus further from the AP threshold, consequently generating states of repolarization or reduction of depolarization in the neuron. Hence, the regulation of the conductance of these kind of channels actively affect the excitability of the concerned cell (Ferrao Santos et al., 2010).

2.2 NEURONAL PLASTICITY IN LEARNING PROCESSES

Across a large variety of animal species, plasticity of intrinsic neuronal excitability is a crucial mechanism for learning and memory formation. Its alteration has been considered an underlying cause of the development of cognitive deficits in the course of aging as well as neurodegenerative conditions such as Alzheimer's disease (Chena et al., 2021).

Dendritic excitability expresses plasticity following various events such as neuromodulation, adaptation, learning and memory, trauma, or disorders (Frick & Johnston, 2005). Alterations

of neuronal intrinsic excitability following a learning experience take place in numerous brain regions, with the goal of enhancing information processing and consolidation through the intensification of synaptic efficiency and plasticity. However, a decrease in baseline intrinsic excitability, along with pathological hyperexcitability have been linked with the presence of cognitive deficits in both normal and pathological cognitive aging.

As previously stated, learning and memory formation rely on physical and chemical mechanisms in neurons, and, while a meaningful contribution is considered to stem from long-term synaptic potentiation, responsible for the reinforcement of synaptic connections and the resulting inter-neuronal communication, synaptic plasticity is not the only process involved in this phenomenon. Intrinsic neuronal plasticity has a role just as crucial. The enhancement of intrinsic neuronal excitability promotes synaptic potentiation and intensification of memory circuits, (Chena et al., 2021) process that has been mainly observed in CA1 pyramidal neurons, where it regulates the efficiency of various hippocampal-dependent associative and learning memory tasks (Dunn et al., 2019). This mechanism acts on memory allocation on a definite ensemble of neurons called engram.

The term 'engram' was coined in the early twentieth century by Richard Semon to refer to the possible physical expression or 'trace' of memory, which he defined as ‘the enduring though primarily latent modification in the irritable substance produced by a stimulus' (Semon, 1921). Research has indicated that memories are encoded initially in a sparsely distributed population of neurons, or a neural 'ensemble'. ‘Artificially reactivating the neural ensemble originally activated during memory encoding leads to memory retrieval. Furthermore, memory allocation, the process of recruiting neurons to form an ensemble representation of the memory, is not random, rather, neurons with elevated excitability have a higher probability of being recruited into a memory ensemble’ (Chena et al., 2021).

Finally, waves of excitability changes in interacting brain areas have the possibility to promote the strengthening of cross-regional synaptic connections facilitating the systems consolidation over longer timeframe contributing to the formation and consolidation of memories (Chena et al., 2021).

Sustained changes in neuronal activity cause subsequent modifications in the expression of voltage-gated ion channels which act on neuronal firing patterns, modulating the cell’s intrinsic excitability. For instance, an upregulation of Na+ channels and a downregulation of K+ channels result in a reduced AP threshold alongside a raise in the number of APs evoked by a depolarization, enhancing the neuronal intrinsic excitability (Frick & Johnston, 2005).

2.2.1 Ion channels

As mentioned above, voltage-gated membrane ion channels are crucial for the modulation of active dendrites properties (Frick & Johnston, 2005).
Several types of different membrane ion channels have been detected and their function has been the focus of many studies. Some of the most important are: potassium (K+) channels, which contribute to the general regulation of dendritic excitability; calcium (Ca2+) channels which determine alterations in calcium concentrations following back-propagating aps and synaptic potentials and sodium (Na+) channels that are involved in AP back-propagation (Johnston et al., 2003).

2.2.1.1 Potassium channels

K+ channels are considered the main regulators of dendritic excitability (Frick & Johnston, 2005).

The four categories of potassium (K+) channels are voltage-gated (A-type) K+ channels, calcium-activated K+ channels, inward rectifier K+ channels, and leak K+ channels.

Latest findings suggest that intrinsic plasticity may be regulated mainly by the A-type K+ channels and calcium-activated K+ channels, which are, specifically the two types of channels linked to AD pathology (Ferrao Santos et al., 2010).

<u>Voltage-gated (A-type) potassium channels</u>

A-type K+ channels can be found in the dendritic area with the role of modulating a voltage-dependent, fast inactivating current, resulting in a reduction of the backpropagation of action potentials into the dendrites. A decrease in this type of current lowers the threshold for LTP induction, affecting this way the synaptic plasticity. Changes in voltage-dependent K+ currents were documented and linked to Aβ production (Ferrao Santos et al., 2010).

Despite each voltage-gated potassium (KV) channel playing a role in the regulation of the membrane potential, two of them have been shown to bring the biggest contribution in the modulation of neuronal excitability: the KV4.2 channel, (Frick & Johnston, 2005) responsible for the fast transient (A-type) K current, and the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel, implied in the transmission of the ‘h’ current (Wijesinghe & Camp, 2011) in the modulation of cellular excitability, rhythmic activity, dendritic integration, and synaptic transmission (Chang et al., 2019).

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels highly influence the firing patterns of neurons and synaptic integration (Frick & Johnston, 2005). They can be found mainly in the heart and in the central and peripheral nervous systems, specifically in the basal ganglia and hippocampus, suggesting a link between their dysfunction and neurodegenerative conditions such as Parknison's and Alzheimer's diseases. Their role is to conduct K+ and Na+ ions, following an activation caused by the hyperpolarization of membrane voltage, and transmit the current, termed If in heart and Ih in neurons, facilitating channel activation at resting membrane potential (Frick & Johnston, 2005).

The membrane potential activation range of the A-type current, or IA, is lower than the one required to fire APs, whereas the 'h' current, Ih, is a slow, non-inactivating current present at resting membrane potential. Both activations result in a diminution of excitatory postsynaptic potentials (EPSPs).

Calcium- activated potassium channels

Calcium-activated potassium channels are important regulators of neuronal excitability. Several activity-dependent changes in intrinsic excitability following learning and memory processes have been detected in association with reductions in the post-burst afterhyperpolarization (AHP), modulated by calcium-activated potassium channel (Ferrao Santos et al., 2010).

The AHP occurs in response to a burst of action potentials that stimulate calcium (Ca2+) influx and the subsequent activation of outer potassium currents leading to the membrane potential turning momentarily more negative compared to the RMP. This process is crucial in regulating neuronal excitability, with enhanced AHPs enabling spike-frequency adaptation, or slowing the subsequent frequency of action potential (Dunn et al., 2019). Depending on their origin or subtype, neurons present various types of AHP (fast, medium, and slow), based on the amplitude and duration of hyperpolarization.

Different subfamilies of calcium-activated potassium channels perform different functions. Firstly, the apamin-sensitive SK channels have small or intermediate K+ conductance, and they are associated with the medium AHP (Ferrao Santos et al., 2010). Inhibition of SK channels results in a reduction of postburst and consequently in an increased intrinsic excitability. In addition to regulating intrinsic excitability, SK channels have also a role in the LTP induction. It has been shown that an activation of SK channels in the hippocampus debilitates associative learning, while their inhibition in the hippocampus, amygdala, and

cortex promoted LTP induction, postsynaptic neuronal excitability, and spatial and non-spatial memories.

A second relevant subtype of calcium-activated potassium channel is the BK channels, which are large conductance K+ channels, responsible for the fast AHP and activated by significant increases in cytosolic calcium levels and voltage changes.

In conclusion, learning-induced changes in ion channels actively modify intrinsic excitability and may function as a further mechanism to facilitate the strengthening of synapses leading to memory consolidation (Dunn et al., 2019).

2.2.1.2 Voltage-gated calcium channels

Voltage-gated calcium channels (CaV), are responsible for the passage of calcium across the membrane as a consequence of membrane depolarization, thus contributing to the intrinsic neuronal excitability under healthy as well as pathological conditions (Frick & Johnston, 2005).

Overall, CaV channels are classified into two main categories: low-voltage activated (LVA or 'T-type'; CaV3) and high-voltage activated (HVA), which, in turns, includes L-type channels and R-type (CaV2.3) channels. L-type channels have been associated with the slow afterhyperpolarization (sAHP), since the blocking of this type of calcium channels has been shown to raise the firing rate of CA1 neurons, and, at the same time, studies have indicated that a decrease in L-type-mediated postburst AHP leads to a boost in the intrinsic excitability, meanwhile the R-type channel is considered to play a crucial role in the processes of synaptic plasticity, and effectively contributes to the afterdepolarization (ADP) in CA1 neurons (Wijesinghe & Camp, 2011).

2.2.1.3 Voltage-gated sodium channels

Voltage-gated sodium channels are strongly involved in the regulation of cellular excitability, their main action is to generate a forceful, inward current in the rising phase of the AP (Frick & Johnston, 2005); playing a role inaction potential propagation and firing frequency (Wang et al., 2016). This kind of channel has been catching the attention of experts since an abnormal expression of it is involved in the alteration of intrinsic neuronal excitability underlying various neurological disorders (Wijesinghe & Camp, 2011).

In conclusion, ion channels play a fundamental role in dendrites' excitability, since, depending on the type, their activation can either increase or reduce signals integration and propagation. This is possible thanks to modulation of the cell intrinsic excitability due to alterations of the influx of Na+ and Ca2+ ions and efflux of K+ ions.
This evidence suggests how intracellular ions concentration would be able to affect intrinsic plasticity of the dendrites, summation of synaptic potentials and finally signal transmission (Frick & Johnston, 2005).

2.3 INTRINSIC EXCITABILITY ALTERATION IN AD

2.3.1 Aβ – AD toxicity

The process at the basis of amyloid-beta AD toxicity is intracellular calcium accumulation, whose severity is determined by the amount of calcium loading and cell defence capability. However, according to recent studies, it has been suggested that these amyloid plaques may not be the cause of the disease but, interestingly, all amyloid forms participate in the alteration of synaptic information processing responsible for neurotoxicity. Indeed, it appears that amyloid toxicity can be generated by small or large oligomeric amyloids even in the absence of amyloid fibres. This hypothesis is supported by findings which shows that AD-like symptoms can be observed in amyloid animal models without any significant presence of amyloid plaques.

The mechanism responsible for these pathophysiological responses can be observed when, upon interaction with the cell membrane, Aβ modifies its conformation to acquire specific functional properties consistent with an ion channel, forming 3D channel-like structures that support its activity. Said ion channels, which usually facilitate the transport of cations, would disturb the ionic homeostasis in the cell. As a result of this disturbance, the cellular calcium concentration is predominantly increased, which, in turn, induces toxicity in the cells.

It is significant to note that large oligomers (ADDLs) not only disrupt in a non-selective manner the viability of the cell membrane but could also potentially be the outcome of aggregation of small oligomers that possess channel-like activity and structural features, contributing, in this way, to their toxicity (Lal et al., 2007).

2.3.2 AD alteration in instrinsic excitability

AD pathology is characterized by tau and amyloid aggregates, which generate lesions that primarily affect the hippocampus and associative cortices, some of the regions where several ion channels, , are widespread, suggesting how they may take part in the aetiology of AD by altering, specifically enhancing, neuronal excitability and Aβ generation (Chang et al., 2019). Tau agglomerates have also been associated to hyperexcitability and enhancement of amyloid-beta induced hyperexcitability (Chena et al., 2021).

Within several ion channels present in the cell membrane, alterations of K+, HCN (Chang et al., 2019) and Na+ channels are strongly correlated with intrinsic hyperexcitability in AD pathology.

Firstly, studies showed that Aβ can downregulate A-type K+ currents, thus promoting neuronal excitability (Wang et al., 2016).

Secondly, the role of HCN channels in Aβ generation has revealed of great interest in latest studies. Evidence suggests that alteration in HCN channels in AD-associated brain regions are strictly correlated with age; notably, differences in the expression of HCN channels and Ih within immature and mature neurons might be responsible for generating changes in neuronal excitability, with effects on neuronal physiology, possibly contributing to the development of a pathological state.

Saito et al. have detected a major decrease in HCN1 levels in the temporal lobe of cynomolgus monkeys during aging, as well as in sporadic AD patients. Moreover, recent findings have shown that HCN1 was enhanced in CA1 pyramidal neurons in both WT and 1-month-old ADTg mice but no presence of it was detected in ADTg mice at 12 and 24 months (Chang et al., 2019).

Moreover, recent studies have indicated the involvement of Na+ channels in intrinsic excitability enhancement, specifically in the initiation and propagation of action potentials in AD conditions (Wang et al., 2016). The distribution of NaV subtypes determines their functions, precisely NaV1.6 subtype is particularly present in the adult CNS acting as an initiator for the action potential. Its activity has, therefore, been shown to highly increase in correlation with Aβ1–42 presence, making it an interesting subject in the study of Aβ pathology (Ciccone et al., 2019).

Evidence gathered on this topic showed that APP is able to promote the surface expression of sodium. For this reason it was investigated the role of Aβ in the regulation of Na+ channels expression. Results showed an overexpression of Na+ channels, notably Nav1.6 subtype, in neurons incubated with Aβ1−42 which may be responsible for a decrease in AP threshold, thus enhancing cellular excitability (Wang et al., 2016). Supporting this view, recent findings displayed how an administraton of riluzole, a Na+ channels antagonist, substantially reduced Aβ1–42-induced hyperexcitability (Ciccone et al., 2019). Additionally, NaV channels inhibition was proved to improve synaptic disorders and cognitive deficits in APP transgenic mice (Wang et al., 2016).

Hence, a selective inhibition of NaV1.6 overexpression and/or hyperactivity could provide a possible therapeutic approach to neutralize hippocampal hyperexcitability and consequent cognitive impairments in early phases of AD.
In conclusion, this evidence supports the view that Aβ pathophysiology may be implicated in the alteration of neuronal intrinsic excitability, and specifically in modifications of ion-channels, typical of the Alzheimer's disease, resulting in the manifestation of cognitive deficits, in particular memory and learning related deficits (Yousuf et al., 2020).

3. ROLE OF HIPPOCAMPUS AND ANTERIOR CINGULATE CORTEX (ACC) IN MEMORY CONSOLIDATION

3.1 LEARNING AND MEMORY

Learning and memory formation are two crucial processes regulated by extensive interconnected neural circuits; these mechanisms are possible thanks to synaptic plasticity and involve several brain regions, namely hippocampus, cerebral cortex, cerebellum, amygdala, and striatum (Roux et al., 2021). The process of memory formation is believed to stem from the activation of hippocampal-neocortical networks responsible for recent memory formation and encoding; over time these neuronal ensembles undergo alterations allowing them to be consolidated within the neocortex resulting in the storage of long-term memory (Kitamura et al., 2017).

The first observations surrounding the mechanisms behind these functions occurred over a century ago, when Ramón y Cajal introduced the idea that synaptic connections alterations may play a role in memory formation. This established the foundation for Hebb's theory: "cells that fire together wire together" which considered synaptic plasticity driven by temporal contiguity of pre- and post-synaptic activity as the process underlying associative learning and memory formation. Finally, subsequent evidence of long-term potentiation (LTP) of synaptic connections following a learning process provided further support for these theories and promoted a deeper understanding of this function (Roux et al., 2021).

'Learning can be distinguished into two forms of memory: declarative and procedural. Declarative memory can be stated and recalled in the conscious mind as an image (episodic information) or language (semantic information). It is typically stored in the medial temporal lobes (MTL), a region that includes the hippocampus (Frankland and Bon tempi, 2005). On the other hand, procedural memory cannot be recalled in a conscious mind or described through language. Synaptic plasticity is important in both declarative and procedural memories (Frankland and Bontempi, 2005; Kreitzer, 2009). However, patients with hippocampal damage and deficits in declarative memory do retain intact learning of certain motor, perceptual and cognitive skills (Squire et al., 2004; Corkin, 2002), suggesting the processes of these two forms are considered separate.' (Goto, 2022)

In order to observe the learning process at a neuronal level, various techniques have been developed which can effectively and reliably investigate the populations of neurons and

brain regions activated during learning. Firstly, a highly efficient tool is the analysis of Immediate-early genes (IEG). These genes, specifically Arc, c-fos, and egr-1, are induced, in particular brain regions during neuronal activity related to learning and can serve as clear markers of this activity. On the other hand, a further, more intrusive, method involves pharmacological interventions on diverse brain regions to either inhibit or enhance memory consolidation (Goto, 2022).

3.1.1 Consolidation theory

Memories in the brain are thought to be stabilized through the process of consolidation. This is considered to happen in parallel both at synaptic and systems levels. Synaptic consolidation takes place quickly and is referred to as the enhanced gene expression and synaptic changes occurring shortly after learning, from minutes to a few hours. On the other hand, systems consolidation takes place over significantly longer time frames, and involves hard-wired modifications within and between nodes of the distributed cortical network. This dual-speed process is believed to enhance the stability of the hippocampal-cortical network.

The process of synaptic consolidation starts with the encoding phase, when reciprocal connections among nodes in a distributed cortical network and the parahippocampal gyrus are induced, possibly mirroring mechanisms of systems consolidation. Initially, the hippocampus serves as a transient storage site for new learning, preserving representations via swiftly yet transitory changes leading to the formation of the first memory traces. It then promotes repeated reactivation of the memory trace, leading to an enhancement of connections in the long-term within other cortical storage areas such as the ACC, which will eventually be able to support remote memory recall autonomously. This gradual development of cortico-cortical connections ultimately responsible for the retention of long-term memory is defined as systems consolidation. The long-term storage of information is believed to be “domain-specific”, suggesting that different sensory cortices are in charge of modality-specific components, or “feature fragments”, associated with previous experiences (Einarsson et al., 2015).

As memory formation proceeds, the reliance on the hippocampus decreases as the function is shifted to the cortex. As demonstrated by Restivo et al. who investigated alterations in spine density following contextual fear conditioning at recent (1 day) and remote (36 day) retention intervals, it was observed that the hippocampus exhibited an increase in spine

density only during the recent retention interval. However, layers II/III of the ACC demonstrated similar results specifically during the remote retention interval.

3.1.2 Systems reconsolidation

Recent research, however, has indicated that retrieval can make remote contextual fear memory, previously independent of the hippocampus, momentarily dependent on it again in a process termed systems reconsolidation. Specifically, studies found that lesioning the dorsal hippocampus caused remote memory impairment selectively following memory reactivation (Einarsson et al., 2015).

Hippocampal reactivation is believed to trigger synaptic activity in the cortex, necessary for the development of the cortical memory trace. This function has been suggested by a study which indicates that the output from the hippocampal dentate gyrus engram, detected via c-fos positive cells, to the medial prefrontal cortex (mPFC) one day after training is necessary for the mPFC dendritic spine density surge in engram cells. Hence, after the primary memory trace formation in the cortex, it is believed that the memory trace is able to mature and consolidate thanks to the reactivation of hippocampal input into the cortex during few days or weeks after training (Goto, 2022).

One hypothesis underlying this mechanism believes that, in order to update the original memory, the hippocampus briefly participates in incorporating new information into the hippocampal-cortical memory trace. Following this process, the hippocampus is no longer needed for the memory's expression, even though it keeps contributing to the memory trace. This observation is in line with the transformation hypothesis, which posits that the hippocampus assists in forming context-specific and detailed memories which, with time, are converted into nonspecific, more schematic representations supported by cortical structures. Disrupting the dorsal hippocampus leads to impaired expression of fear memory that is specific to a certain context, but not the generalized memory acquired.

To examine the role of the ACC and the extension of the hippocampus involvement in the reactivation of contextual fear memory, Einarsson et al. practiced pharmacological suppression of neural activity in these areas. First, results showed that suppression of activity in the ACC leads to memory expression impairment solely when assessed 24h, but not 6h, after reactivation. Secondly, only combined suppression of neural activity in the ACC and dorsal hippocampus disrupted memory expression when tested six hours later. These

findings suggest that either structure may support recent recall or remote memory recall independently (Einarsson et al., 2015).

3.1.3 Recent and remote recall

Previous studies have focused exclusively on the overlap between the encoding neuronal ensambles and the recent or remote recall neuronal ensambles and have reported a considerable reactivation of the two. However, it remains unclear whether the hippocampal neurons activated during remote and recent recall are the same, or whether they overlap only partially with the encoding engram cells, but not with each other.

When examining the alteration in recall engrams between the two time periods, it is crucial to comprehend the dynamics of long-range connectivity within diverse brain regions. Structurally, connections between two separate brain areas do not change, but the population of cells that make up an engram can vary, perhaps due to their specific projection target. A study by Rafaeli et al. showed that the projection of dCA1 engram cells to the ACC rises as memory ages, which indicates that new neurons incorporated in the remote engram are more likely to be ACC-projecting neurons. Moreover, CA1/ACC cells were proved to be functionally involved, since inhibiting this projection during systems' consolidation within recent and remote recall resulted in remote memory being compromised (Refaeli, Kreisel, Groysman, Adamsky & Goshen, 2023). These findings align well with the role of hippocampus in the process of system reconsolidation aforementioned and the involvement of ACC in remote memory formation (Refaeli et al., 2023).

3.2 ANTERIOR CINGULATE CORTEX (ACC)

A significant region identified by Broca in the limbic lobe is the cingulate cortex, which encircles the corpus callosum. Broca adopted the term limbic to describe structures that are positioned at the periphery or border (the literal meaning of limbic) of the hemispheres. Within the several limbic structures, besides the cingulate cortex, we can find the hippocampus, and the amygdala.

The anterior cingulate cortex (ACC) is a region situated around the anterior third of the cingulate cortex, connected with a number of other limbic and associated areas, notably the amygdala and the orbitofrontal cortex (OFC), which are involved in emotion and reward-

related processing, and the hippocampus, involved in memory formation (Rolls E., 2019). The ACC, specifically its caudal area, is part of a distributed cortical network related to the consolidation and the recall of remote memory, as demonstrated by several studies (Bontempi et al., 1999),(Frankland et al., 2004), (Teixeira et al., 2006). As mentioned above, it has been shown that interfering with the ACC activity at different moments during learning selectively affects recall of remote memory, whereas recollection of remote memory is linked to enhanced expression of different IEGs, markers of neuronal activity, and GAP-43, a spinogenesis indicator, in this area (Weible, 2013). Finally, the capacity to recollect distant memories is correlated to an increment in the size and density of spines on layer II/III pyramidal neurons, structural modifications that become apparent as soon as a week after training.

3.2.1 Associative learning in ACC

Strong evidence has demonstrated the involvement of the ACC in associative learning (Buchel et al., 1999), (Knight et al., 2004), (Kronforst-Collins et al., 1998). First, it has been shown that lesioning the ACC during trace fear conditioning interfere with learning, resulting in a decreased occurrence of freezing behaviour (CR) towards a tone (CS) linked to a foot shock (US). (Han et al., 2003) Secondly, training produced a raise in c-Fos expression in the ACC area, indicating a surge in neuronal activation, offering valid data supporting the conclusion that the ACC plays a significant role in building the CS-US connection (Clark et al., 2002)

Subsequent studies have been carried out specifically focusing on the involvement of ACC in contextual fear conditioning paradigm on mice subjected to recent and remote memory recall testing. The results showed that the mice exhibited strong and sustained freezing behavior at both retention intervals, indicating a strong association between the cage and the aversive footshock stimulus, thus confirming the successful learning. The expression of the immediate early genes (IEGs) was increased in area CA1 of the hippocampus at the recent, but not remote, retention interval. The opposite trend was found in the ACC with enhanced IEGs at the remote retention interval.

In conclusion, these findings support the view that considers the ACC a region that plays a crucial role in processes of learning and memory consolidation, with a particular focus on associative learning and remote memory retrieval.

3.2.2 Additional memory-related functions of ACC

Besides memory and learning, the ACC has also been associated to numerous other functions supporting these processes, such as attention, action/outcome valuations, novelty detection, determination of salience, premotor planning and movement execution, processing of pain, error detection, monitoring or resolving conflict, mediating adaptation to changes in cognitive load, processing of reward and decision making.

The data indicate that ACC-mediated attentional mechanisms and motor processes, such as those regulating gaze control, would promote the learning and recalling of which elements in the environment to focus on and which to ignore, and would optimise the conditions for fast integration of new information. Lesions in this area hinder the ability to effectively prioritise attentional resources, resulting in impairments in memory encoding and remote retrieval (Weible A., 2013).

As mentioned above, a further function mediated by the ACC which, in turns, contribute to memory and learning, is the action/outcome valuation. The anterior cingulate cortex is connected to the orbitofrontal cortex and amygdala, structures which send information about value of the outcome of actions, based on the discrimination between reward or punishment. This function is crucial for learning the action necessary to obtain a reward or avoid a punisher through a process called ‘action–outcome learning’ (Rolls E., 2019).

3.2.3 ACC in ageing and AD

ACC is one of the most severely affected areas in AD pathology. Alterations in metabolism and functional connectivity within this region crucially contribute to the development of cognitive impairment, which is associated with ageing and dementia. Ren and colleagues (2018) have suggested that Aβ promotes hyperexcitability of pyramidal neurons in the ACC. This is the result of increased dopamine release from dopaminergic axons, leading to a significant inhibition of presynaptic GABA release, eventually resulting in an excitatory/inhibitory imbalance in the ACC. Hence, modifications in neuromodulatory networks within the ACC caused by Aβ may contribute to cognitive deterioration in AD.

Additionally, a further Aβ-induced effect comprised structural synaptic alterations and impaired synaptic transmission. AD-related modifications of neuronal activity and dendritic spine loss, dysgenesis and instability impact the ACC, leading to alterations in synaptic plasticity and eventually deficits in memory formation (Cuia et al., 2022). Finally, evidence

has proved that ACC volume loss is a reliable indicator of aMCI progression to AD (Yuan et al., 2022), and that presence of NFT pathology in this area exhibits a correlation with symptoms like agitation and apathy observed in AD (Tekin et al., 2001).

Therefore, these studies show the significant contribution of the ACC in the cognitive impairment related to AD development and highlight the importance of a deeper understanding of its role in the pathology.

3.3 HIPPOCAMPUS (HPC)

The hippocampus is a structure that lays in the medial temporal lobe (MTL). It is composed of a ventral (or anterior) part, connected to the hypothalamus and amygdala, contributing to the emotional components of memory processes, and a dorsal (or posterior) part, connected to dorsal lateral septum and the mammillary body and linked to spatial memory processing. It is then possible to identify four distinct regions, namely, the dentate gyrus (DG), and the three subfields of cornus ammonis (CA) CA1, CA2 and CA3 (Roux et al., 2021). The hippocampus receives inputs from cortical areas, specifically from the dorsal stream about space, action, and ‘where’ events occur, as well as the ‘what’ happens from the ventral processing stream, making it a key structure in the integration of information and in the encoding and subsequent retrieval of new long-term episodic memories (Rolls E., 2019).

At the start of the twentieth century, the location of memory in the brain was the subject of great research but it was not until the case of patient H. M. in 1957 that localization of this function was partially accomplished. H.M. suffered from intractable epilepsy; thus, to alleviate his symptoms, a bilateral surgical resection of his medial temporal lobe was carried out. As a result of the procedure, he experienced a severe anterograde and temporally-graded retrograde amnesia, although his other cognitive abilities remained intact. At the time, there was no certain notion regarding the structures at the basis of the damaged functions since the area that had been removed included various regions, notably hippocampus, parahippocampal gyrus, uncus and amygdala, nevertheless, this case is considered the first clear evidence of a specific brain structure being disproportionately involved in memory processes. The fact that only recent memory was selectively impaired, while the remote memory was speared, shed light on the possibility that long-term storage

of information, also known as consolidation, might selectively take place elsewhere in the brain (Weible A., 2013).

3.3.1 System consolidation

The hippocampus plays a fundamental role in the first encoding phase of memory consolidation, nevertheless, after being acquired, memories that rely on the hippocampus undergo a process of systems consolidation. This process enables memories to become independent of the hippocampus and reliant on the anterior cingulate cortex (ACC) for memory expression. However, it is worth noting that consolidated remote memories can return to being temporarily dependent on the hippocampus again following memory reactivation in a process, called system reconsolidation, discussed in previous paragraphs (Einarsson et al., 2015).

3.3.2 Hippocampus in ageing and AD

Until this point it has been mainly discussed a healthy functioning of the hippocampus; nonetheless, it is to be noted that, among a wide variety of additional brain modifications, structural and functional alterations in this structure have been considered crucial contributors of cognitive decline. Consequently, magnetic resonance imaging (MRI) has become one of the primary measures, combined with cognitive scales, employed in ageing studies or in clinical practice to assess the impact of brain disease. Hippocampal atrophy is considered one of the most revealing factors associated to aging and to neurodegenerative and psychiatric disorders, with accumulation of neurotoxic Aβ in vital regions of the hippocampus being identified as a possible cause of this process. The total volume of the hippocampus is, indeed, thought to decrease at a rate of about 1.5% per year after the age of 70 and hippocampal volume loss is believed to reach 10-15% in patients with mild cognitive impairment (MCI) (Roux et al., 2021).

Compared to normal ageing, individuals with AD exhibit a more accelerated loss of volume in the hippocampus, the region where neurofibrillary tangles (NFTs) first appear (Thompson et al., 2004) this atrophy has been observed even before the diagnosis of dementia (Laakso et al., 1999).

Additionally, AD has also been associated with a reduced hippocampal functional connectivity. Allen et al. revealed through functional connectivity magnetic resonance imaging (FCMRI) a reduced functional synchrony within the left and right hippocampi in

patients with AD. Moreover, the study also discovered a notably limited pattern of functional connectivity between the hippocampus and neocortex, with a complete absence of connectivity observed with the frontal lobes.

These alterations potentially play an important role in cognitive impairment associated with AD, making it an important point to further investigate this area.

4. RESULTS

4.1. RESULTS

Aβ oligomers are structures formed by the aggregation of misfolded Aβ peptides, produced by APP, which trigger a state of neurotoxicity in the brain. This is caused by an increase in intracellular calcium concentration, disrupting ionic homeostasis and eventually pathologically altering the intrinsic excitability of the cell. It is hypothesized that changes in neuronal excitability play a profound role in memory formation which leads us to believe that their alteration may be an underlying mechanism of early memory impairment in AD. Based on this information we decided to investigate the role of Aβ in aberrant intrinsic excitability, correlated to memory formation perturbations in mouse-model of AD, with a specific focus on engrams in the ACC, crucial for memory consolidation, remote memory recall and associative learning.

To achieve this goal, we divided our project into three main sections:

(1) Behavioural and histopathological characterization and validation of a 3xTg-AD mouse model of AD

(2) Identification of the reorganization of neuronal ensambles (engrams) associated with memory impairment following plasticity-inducing paradigms; and

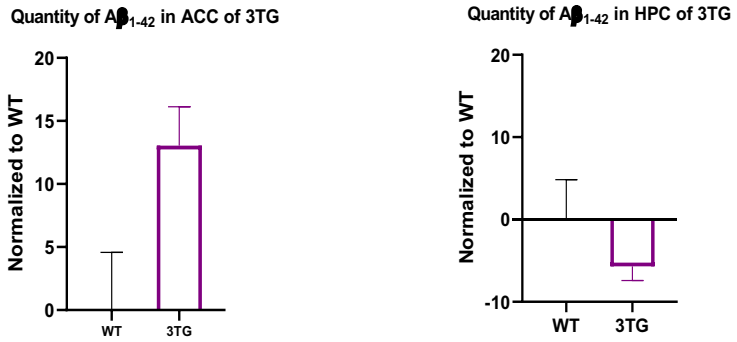
(3) Analysis of the alteration of intrinsic excitability in HPC and ACC in early stages of AD.

4.1.1. Histopathological and behavioural characterization and validation of a 3xTg-AD mouse model of AD

4.1.1.1. Histopathological characterization

We used a triple transgenic mouse model of AD, modified to express human APP and presenilin gene with the respective mutations, contributing to accumulation of Aβ peptides and eventually a misfolded tau protein. These mutations allow the 3xTg-AD model to reproduce several key aspects of the human pathology of AD. We use the animals at the age where the intracellular accumulation of Aβ oligomers (Aβo) is expected, before the outcome of tau pathology (https://www.alzforum.org/research-models/3xtg). Our study

focuses on the effect of Aβo within the ACC on memory consolidation processes, so we quantified the amount of soluble $A\beta_{1-42}$ in ACC by using ELISA, an assay specific to



recognize the peptide form of human $A\beta_{1-42}$.

Figure 1. Quantification of $hA\beta_{1-42}$ peptide in the ACC (left) and HPC (right).

Elisa analysis was conducted to quantify the extent of Aβ accumulation in the ACC and hippocampus of four-month-old 3xTg-AD mice standardized to the wild type line. Results revealed a significantly higher amount of Aβ deposits in the ACC of transgenic mice (t-test, p=0.0453, n=5 per genotype) while no significant difference was detected in the hippocampus (t-test, p=0.2996,n=5 per genotype, figure 1). These data show that the accumulation of Aβo in early stages of the AD starts in neocortical regions (such as ACC) and the effect of this accumulation can be an underlying mechanism for early stage symptoms.

Furthermore, western blot analysis was used to assess the presence of AD hallmarks: Aβ accumulation and others such as: hyperphosporylated tau tangles and glial inflammation marker GFAP. Analysis were performed using a fresh ACC tissue of 3xTg-AD mice at the age of 3-4 months, but also old (14-15 months) mice as a positive control (Figure 2). Analysis in old mice detected presence of hAPP in the 3xTg-AD line but not in wild type one, validating the model. Possibly due to a contamination of the brain samples with mouse immunoglobulins we were not able to visualize the Aβo. Further, we observed slight but not statistically significant difference of GFAP in old 3xTg-AD mice. GFAP is an astrocytic cytoskeleton intermediate filament protein. Increase of the GFAP suggest an increase in reactive astrocytes further contributing to neuroinflammatory changes characteristic for human AD. The expression of GFAP is usually higher in areas surrounding Aβ plaques and increased with tau accumulation in the brains of patients with AD. However, we did not

observe any changes in Tau pathology (total Tau vs phosphorylated Tau) at this age (14-15 months). We did not observe any changes in Tau or GFAP in young mice.

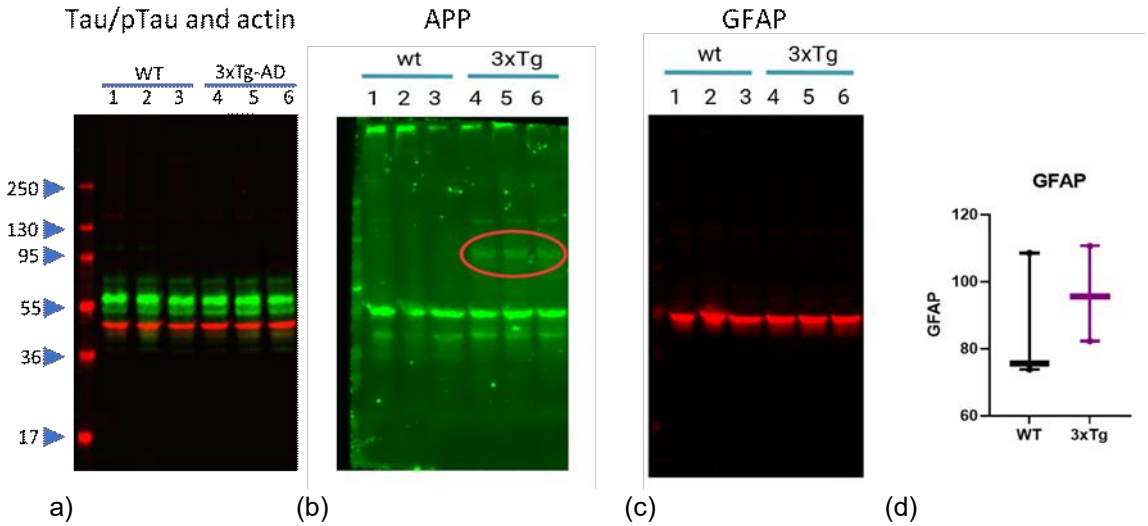


Figure 2: Western Blot analysis in old (14-15 months) wild type and 3xTg-AD mice. (a) Tau-5 (55 kDa) in green and actin (42kDa) in red detection. (b) APP deposits (130kDa) circled in red detection. (c) Glial inflammation marker GFAP (50kDa) detection (d) GFAP levels in wt and 3xTg mice.

4.1.1.2. Behavioral characterization

To assess associative learning and memory formation impairment we employed contextual fear conditioning paradigm (CFC) followed by a phase of memory retrieval either at recent (day one, D1), or remote, (day fourteen, D14 or day twenty-one, D21) intervals. Learning was deemed successful if the mouse associated a context (a cage in our case) with a

stimulus (a footshock) and memory formation was quantified by measuring the freezing response during memory retrieval triggered by the same context at different time points.

Data indicated no significant difference in the freezing response between the two genotypes at recent interval (D1) or after the last shock of acquisition (D0). However, significant differences were observed in remote memory retrieval, displaying reduced recall performances in D14 and D21 of transgenic mice compared to wild types. (Figure 3).These findings demonstrated that memory encoding was spared whilst long-term memory consolidation was impaired in 3xTg-AD mice. This is consistent with evidence indicating the ACC involvement in consolidation and retrieval of remote, rather than recent, memory retrieval, thus confirming the presence of Aβ-induced perturbation of ACC activity in AD pathology. In conclusion 3xTg-AD line accurately reflected the progression of human cognitive impairments in early stages of the disease.

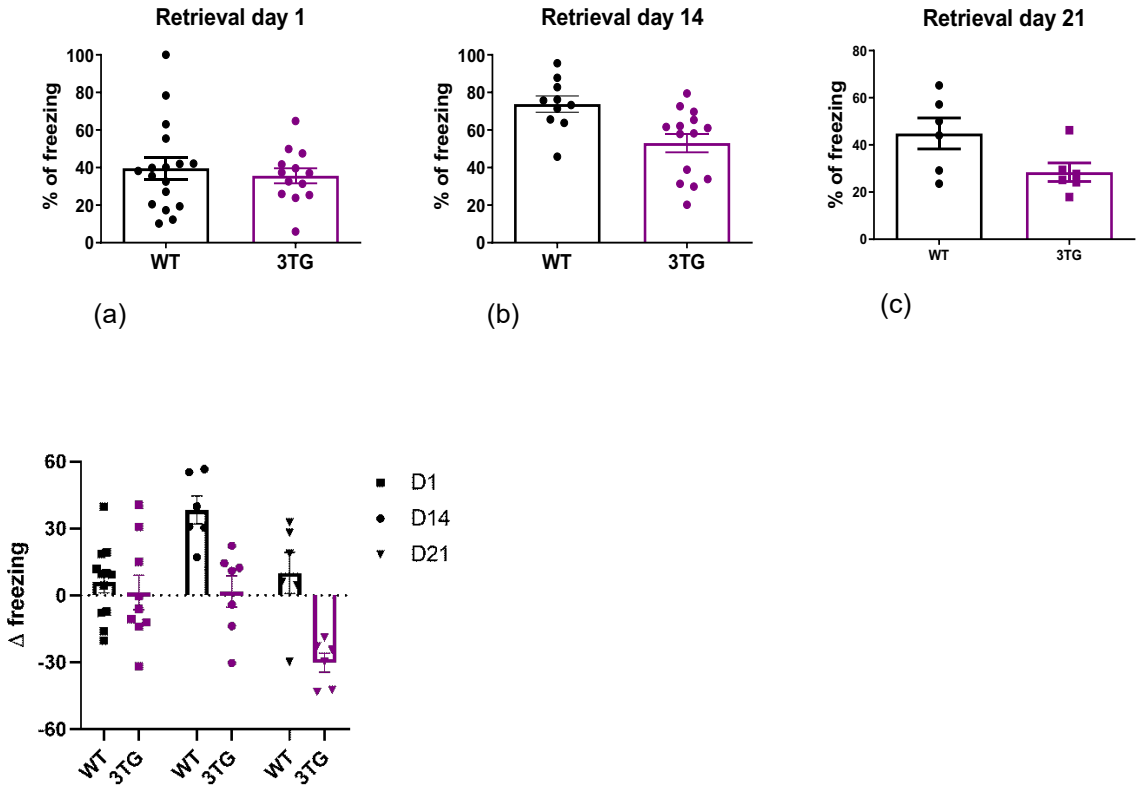


Figure 3: Freezing response in CFC in wild type and 3xTg mice. (a) % of freezing response at retrieval D1. (b) % of freezing response at retrieval D14. (c) % of freezing response at retrieval D21. (d) Variation of freezing response between the genotypes at D1, D14 and D21.

Therefore, evidence collected through Elisa, Western Blot and CFC analysis confirms the suitability of 3xTg-AD mouse model for our project as a representative animal model for AD by displaying an accurate progress of the disorder, comprised of Aβ accumulation associated with cognitive impairment at early stages and probable later development of tau tangles.

4.1.2. Identification of the reorganization of engrams following plasticity-inducing paradigms

We aimed to examine the reorganization of memory circuits in the ACC following plasticity-inducing paradigms associated with memory impairment.

This was accomplished using the tattagging system, a technique that allows us to identify and access the subset of neurons engaged in a specific task, which in our case was a

learning-related activity. It was possible, through image analysis, to detect markers of neuronal activity involved either in memory encoding or retrieval. The activation of the two populations was then colocalized to observe their reactivation.

As a plasticity-inducing memory task we relied once again on the CFC paradigm. A subset of the mice that had previously undergone this task were used for engram reorganization image analysis in the different phases (marked with the lighter color in the graphs. Figure 4)

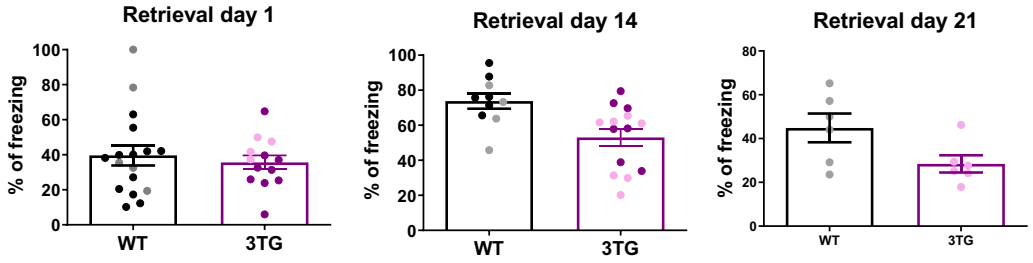


Figure 4: Freezing response in CFC in wild and 3xTg mice at three different time points. It is possible to see the subset of the mice whose engram reorganization proceeded to be studied (light grey and light pink)

Our main interest in this stage was the investigation of the engram activity in the ACC during a learning process.

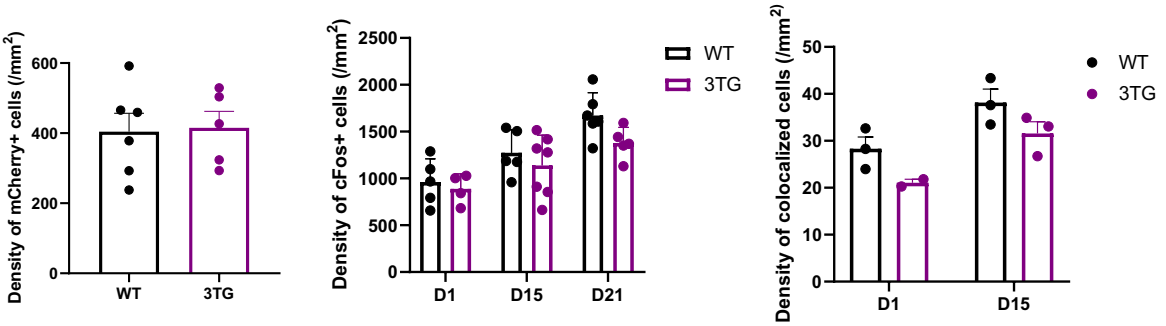
To do so we first focused on neuronal activation in the encoding phase, expressed during CFC task and identified by the long-term expression of IEG-gene mCherry. Results showed no significant differences between populations, suggesting that the tagging of the ACC during encoding is not affected by the AD pathology.

Secondly, we observed engram reactivation through memory retrieval task at three time points: recent (D1) and remote recall (D14 or D21), revealed by the expression of IEG-gene cfos. Results showed a significant difference within the same genotype in the number of neurons activated at recent and remote retrieval, with the ladder displaying higher values, thus confirming the role of ACC in long-term memory retrieval (or recall). Moreover, no significant difference in the neuronal activation was detected between the genotypes in all retrieval intervals, indicating that long-term memory impairment we observed was not due to changes in number of neurons engaged by the retrieval of the memory.

Finally, we colocalized the neurons active during memory encoding and retrieval to observe the reactivation rate of engrams. These data could show us if there is the problem in the access to the ‘learning/encoding’ engram during the long-term memory recall. Data revealed

no significant differences between the genotypes in the populations of neurons that were reactivated, suggesting that the cause of memory deficits might not rely on the alteration of a neuronal reactivation rate, but rather on a different mechanism. (Figure 4).

Since studies have shown that memory formation and consolidation rely on neuronal plasticity, promoted by changes in intrinsic excitability of interacting brain areas, and that pathological hyperexcitability has been associated with memory deficits in both healthy and pathological cognitive ageing, aberrant intrinsic excitability induced by Aβ has been considered an interesting candidate as a mechanism underlying memory impairment in AD.



(a) (b) (c)

Figure 5: Investigation of the engram activity in the ACC during a learning process identified by the expression of IEG-genes. (a) Neuronal activation in the encoding phase during CFC task expressed by IEG-gene mCherry. (b) Engram reactivation through memory retrieval task at recent (D1) and remote (D15) retrieval expressed by IEG-gene cfos. (c) Colocalization of neurons active during memory encoding and retrieval.

4.1.3. Analysis of the alteration of intrinsic excitability in the ACC in early stages of AD

Initially, the electrical properties of neurons were characterized using whole-cell patch-clamp technique. Secondly, said neurons were reconstructed using Neurolucida software and Confocal microscopy to verify their inclusion in the target population and to potentially identify differences between the two genotypes. The analysed mice were aged between 18 to 20 weeks and channel blockers were administered to isolate the neuronal activity.

We first started by studying passive electrical properties of neurons. In the 3xTg population, resting membrane potential (RMP) exhibited significantly greater values (t-test, p=0.0453, n=4 per genotype), alongside input resistance showing higher values albeit not reaching the statistical significance threshold (t-test, p=0.1268, n=4 per genotype). This indicated an enhanced excitability of these neurons compared to wild type line. We then proceeded with active properties and found that the number of evoked action potentials (Aps) (2 way anova, p=0.0007 n=4 per genotype) and rheobase (t-test, p=0.0388, n=4 per genotype) was significantly higher in the transgenic line, whereas transgenic inter spike interval (ISI) adaptation values were lower however they did not reach the statistical significance threshold (t-test, p=0.1248, n=4 per genotype), thus suggesting once again an increase in intrinsic excitability in 3xTg mice. Furthermore, the maximum rate of action potential depolarisation (AP max dV/dt) was significantly lower in transgenic mice (t-test, p=0.0421, n=4 per genotype), suggesting greater excitability. Additionally, we analysed AP threshold (t-test, p=0.5593, n=4 per genotype), AP halfwidth (t-test, p=0.3350, n=4 per genotype) and AP amplitude (t-test, p=0.3348, n=4 per genotype) but these data didn't provide any difference between genotypes. (Figure 6).

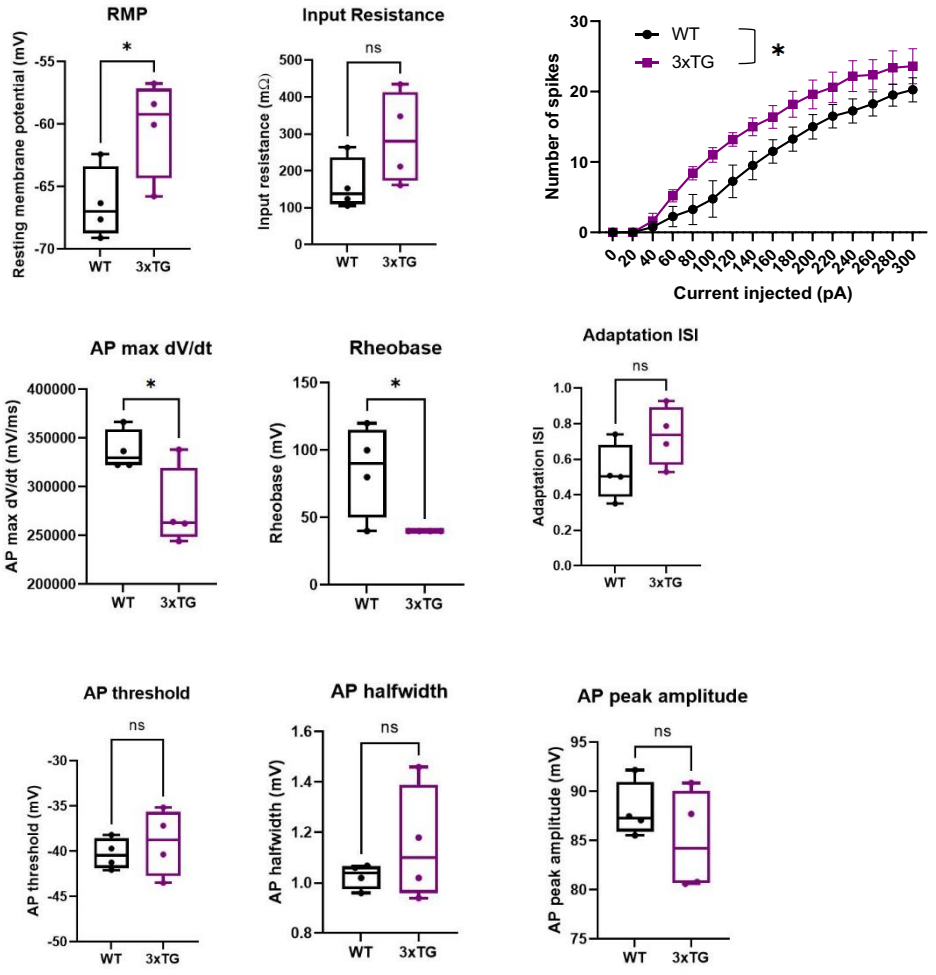
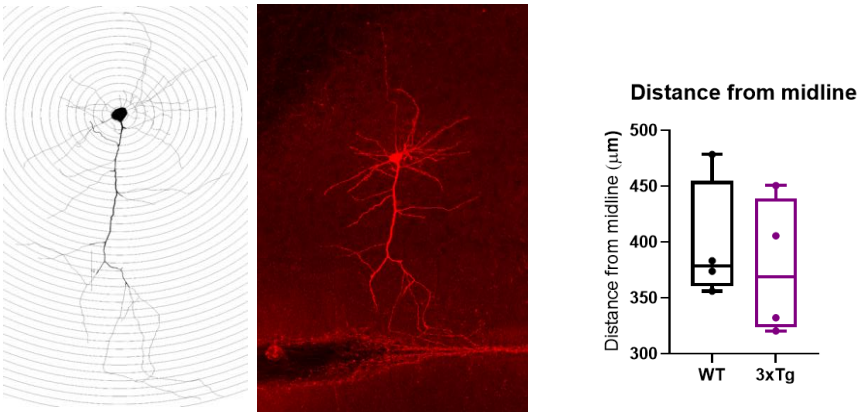


Figure 6: Analysis of electrical properties of neurons in wild type and 3xTg mice. (a) resting membrane potential values. (b) Input resistance values. (c) Number of evoked APs in response to gradually increasing current injections. (d) maximum rate of action potential depolarisation (AP max dV/dt) values. (e) Rheobase values. (f) ISI adaptation values. (g) AP threshold values. (h) AP halfwidth values. (i) AP peak amplitude values.

Finally, our target population comprised of Layer V thick-tufted neurons in the ACC as they constitute the primary output of the ACC. We structurally reconstructed the recorded neurons to verify their type considering their morphology and distance from the midline (Figure 6), and we further analysed the structural properties of apical dendrite length, primary dendrite numbers, and number of nodes and tips to compare the two genotypes. (Figure 7). Firstly, findings validated the classification of neurons in the correct population. After this, we compared the structural properties of the two lines and found no significant difference: analysing apical dendrite length (t-test, p=0.4370, n=4 per genotype), number of primary dendrites (t-test, p=0.1243, n=4 per genotype), number of nodes (t-test, p=0.8018, n=4 per genotype) and number of ends (t-test, p=0.9781, n=4 per genotype). These results supported the view that alterations in neuronal morphology/structure might not be the origin



(a) (b) (c)

of functional alterations in the 3xtg line, further validating the involvement of changes in intrinsic excitability in Aβ pathology.

Figure 7: Acquisition of neuronal morphology data. (a) Neuronal recontruction via Neurolucida software. (b) Neuronal image obtained via Confocal microscopy. (c) Distance of the soma from the midline in wild type and 3xTg mice.

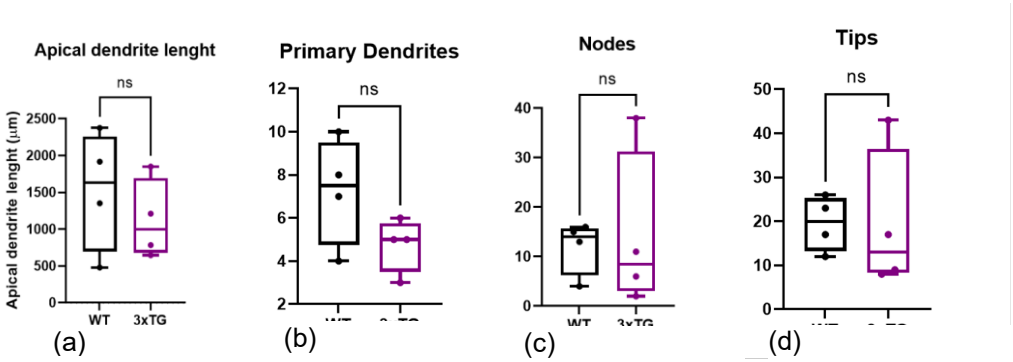


Figure 8: Structural neuronal analysis of wild type and 3xTg mice. (a) Apical dendrite length values. (b) Primary dendrites number. (c) Nodes number. (d) Tips number.

4.2. DISCUSSION

AD is a disorder that affects a growing proportion of the population, resulting in implications for the patient, their caregivers, and the healthcare system. Although Aβ plaques have long been considered a primary contributor to the cognitive impairment associated with AD, in recent years, a theory has gained traction in the scientific community proposing that deficits may actually arise from the accumulation of Aβ oligomers, prior to plaque formation. Aβ oligomers have been demonstrated to play a vital role in the pathological alteration of neuronal intrinsic excitability, making them a strong candidate as an underlying mechanism of early cognitive deficits in AD. One of the first functions affected by Aβ pathology is memory formation, specifically memory consolidation. This project focuses on long-term memory impairment and activity alteration of the ACC, specifically Layer V thick-tufted neurons in a population of AD mouse model (3xTg-AD) compared to a wild-type line (3xTg-wt).

First, we applied a CFC paradigm to investigate associative learning. The recordings showed no significant differences in freezing response at D1, but significant differences were observed at D14 and D21 between the genotypes. These findings demonstrated that Aβ pathology affects remote, but not recent, memory recall in transgenic mice. Furthermore, an overall increase in freezing response in remote intervals confirmed the role of ACC in memory consolidation. These findings were corroborated by the data gathered through analysis of engram activation during memory formation.

While encoding memories in CFC, there was no substantial variation in the number of activated cells in the ACC between the genotypes. However, during retrieval, marked differences were observed in the neurons population activated between the recent and remote recall within the same genotype, whereas no significant differences existed between the genotypes during recent memory recall. Further analysis of reactivation between encoding and retrieval revealed no significant differences between the populations, indicating that the cause of memory deficits in Alzheimer's disease may not be attributed to the alteration of reactivation rate.

Finally, a study of individual neurons indicated no substantial differences in morphology between the two lines using Neurolucida and Confocal analysis. However, functional differences in intrinsic excitability were observed using electrophysiological recordings, with a general tendency towards higher excitability in the transgenic population. These findings are consistent with past evidence indicating that intrinsic excitability could have a critical function in memory processes. Furthermore, this study suggested that differences in performance between the populations might be due to functional, rather than structural variations, hence, specifically hinting at Aβ-induced hyperexcitability as an underlying mechanism of cognitive impairment in AD.

4.3. CONCLUSION

In conclusion, this study shows that Aβ accumulation in ACC early on in the pathology does not affect the proper learning of a memory. Additionally, evidence proves that ACC is involved in remote memory consolidation. Furthermore, findings suggest that engram reactivation during retrieval does not play a role in those AD alterations, hinting that early AD-related functional impairment may result from intrinsic excitability changes.

Based on this information, a promising approach for future research would be to try to identify the ion-channels involved in early memory consolidation impairment investigating their up or down-regulation through analysis of the RNA obtained from cytosol extraction. Additionally, it would be intriguing to modulate their activity by interference or by the use of transgenic animals.

5. MATERIALS AND METHODS

5.1 ANIMAL USE

In this study, we used mice as an animal model to investigate neocortical engram reorganization during long-term memory consolidation and impairment in Alzheimer's disease (AD). Mice are commonly employed in neuroscience research due to their easily editable genome, rapid reproductive cycle, and human-like central nervous system.

5.1.1 Ethical considerations

Before starting our experiments, we took into account the ethical considerations related to the use of animals for research purposes. All animal procedures, including anesthesia and surgery, were performed in accordance with European guidelines for the care and use of laboratory animals. The experiments carried out have received the approval of the animal welfare monitoring committee of the University of Bordeaux and the National Institute of Health and Medical Research (Inserm) in France. The number of animals used was minimized and their suffering minimized during the experiments. In addition, the researchers involved in this study received training in animal experimentation (according to decree no. 2013-118 of February 1, 2013) to handle the animals and ensure their well-being.

5.1.2 Mice used for experiments

The mice used in this study are 3xTg-AD models (Oddo et al., 2003),carrying three mutations associated with familial Alzheimer's disease: APP Swedish (amyloid precursor protein), MAPT P301L (tau protein) and PSEN1 M146V (presenilin 1). These mutations allow the 3xTg-AD model to reproduce several key aspects of the human pathology of AD: a progressive accumulation of amyloid plaques in the brain, hyperphosphorylation and abnormal aggregation of tau protein (neurofibrillary tangles), and cognitive impairments in learning and memory task. These mice exhibit progressive Aβ accumulation and extracellular Aβ deposits appearing from six months in the frontal cortex. Tau alterations are detected later, between 12 and 15 months, in the form of conformational and hyperphosphorylated aggregates in the hippocampus. We used mice at the age of 3- 4

months, during the early stages of the disease development, when there is no reported neurofibrillary tangles but only increased reactivity of intracellular Aβ, allowing us to dissect the contribution of Aβ to memory formation and underlying engram reorganization.

The 3xTg-AD mice come from a mixed C7BL/6;129X1/SvJ;129S1/Sv genetic background. They are available from The Jackson Laboratory under stock number 034830.

For the experiments we used two distinct groups: wild-type, 3xTg-WT (WT) mice and 3xTg-AD (3Tg) model mice. The mice were evaluated at three different time points, namely day 1 (D1), day 15 (D15) or day 21 (D21).

5.2 VIRAL INJECTIONS

Two weeks before the behavioral experiments, bilateral stereotaxic injections were performed in the anterior cingulate cortex (ACC) coordinates : AP +0.9 mm, ML± 0.75 mm, DV -1.65 mm, 20° from a vertical line. Mice were anesthetized with 4% of isoflurane, then transferred and placed on a heating mat in a stereotaxic frame with a continuous supply of isoflurane during all surgical procedures. For analgesia, mice received subcutaneously 0.1 ml of buprenorphine solution (0.03 mg/ml). Before incising the skin on the skull, a local anesthetic, lidocaine (0.1 mg/ml) was subcutaneously injected at the site of surgery.

Mice were bilaterally injected with 500 nl (150nl/min) of AAV-cfos-tTA and AAV-TRE-mCherry (1:1 ratio) used for neuronal labeling in CFC experiments (**Figure 2A**). After virus infusion, the needle was held at the injection site for an additional five minutes to allow sufficient diffusion of the virus. After surgery, the mice were kept in a new cage in a heated box until they woke up.

5.3. BEHAVIORAL EXPERIMENTS

5.3.1. Contextual fear conditioning (CFC)

Before the behavioral testing, mice were handled daily for five consecutive days by the experimentator. 48h before CFC doxycycline food was replaced with the regular food.

Doxycycline food was given back to the mice immediately after exposure to the CFC (Figure 2B).

On the day of CFC, the mice were taken to the acclimation room for at least 10 minutes. Subsequently, they were transferred to the CFC room in which there was an isolation box with a conditioning chamber inside. The conditioning protocol was as follows: three minutes of free exploration then three one-second shocks of 0.5 mA, spaced 1 minute apart. After the last shock, mice remained in in the box for additional 60 seconds. Finally, the mice were placed in a home cage to avoid any communication with those who have not yet passed through. Between each pass, the box is cleaned with water and 70% ethanol. A video camera was placed above the conditioning box allowing the observation and recording of the behavior of the mice.

5.3.2. Memory Retrieval

To assess memory retrieval, mice were placed back into the same CFC box used during the training phase (Figure 2B). Before the start of the test, the mice were transported to the waiting room (acclimatization for at least 10 minutes). Then they were transferred to the fear conditioning room. In this phase of memory retrieval, no electrical stimulation was applied. The mice were exposed to the same context as during the CFC for a period of 6 minutes. Freezing behavior, an indicator of fear memory, was recorded and analyzed. The time spent freezing was measured for 6 minutes.

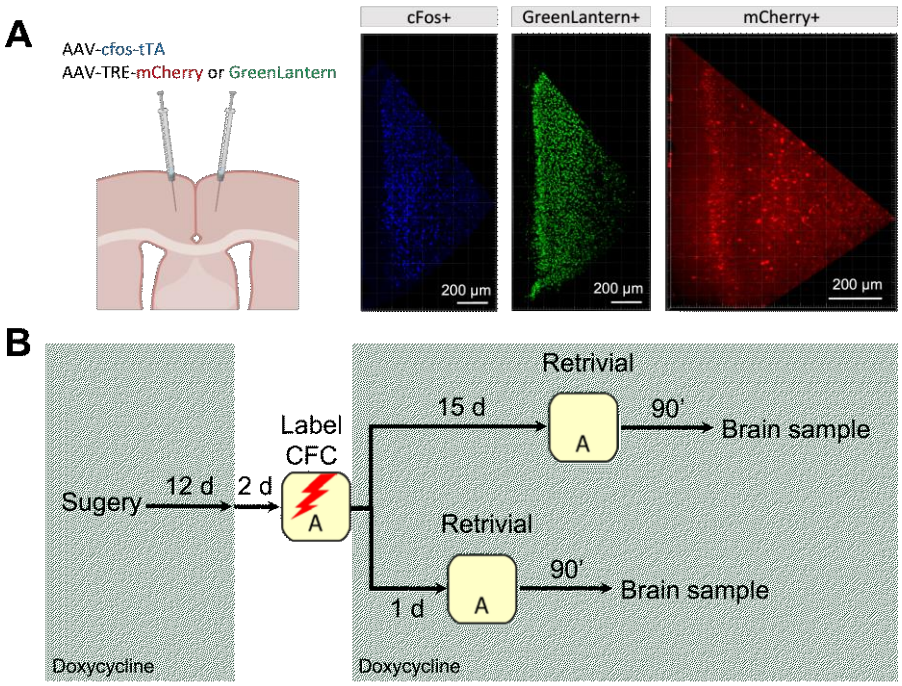


Figure 2: Steps of the experimental protocol. (**A**) Neurons in ACC bilaterally injected with AAV expressed mCherry (red) or GreenLantern (green) and cFos neurons marked with fluorochromes (blue) (**B**) Diagram of the experimental design.

5.3.3. Assessment of memory performance

In order to evaluate the memory performance of mice, we measured the freezing behavior during the retrieval. The retrieval was carried out 1 or 15 days after the conditioning of the contextual fear. An increase in freezing behavior is considered an indicator of successful contextual memory, while a decrease in freezing behavior suggests impaired contextual memory (see limitation). The results were analyzed and compared between different groups of mice to assess the impact of different experimental manipulations on memory retrieval. The mice were considered in freezing when they have no movement except breathing. Data are expressed as a % of time mice spent freezing.

5.4. EUTHANASIA AND BRAIN EXTRACTION

To be able to study the neurons engaged during memory retrieval, the mice were sacrificed and perfused within 90 minutes of the retrieval experiment. They were first anesthetized using an isoflurane induction chamber for about three minutes. They were then euthanized by lethal intraperitoneal injection of Exagon® in a mixture with Lidocaine. The injection dose was 0.001 ml per gram of body weight for mice. Next, an intracardiac infusion into the left ventricle of 1M phosphate-buffered saline (PBS) was performed to evacuate the blood, followed by an infusion of 4% paraformaldehyde (PFA), a fixing agent. The animals were then decapitated. Immediately afterwards, the brains were removed gently to avoid tissue damage. Brains were fixed in 4% PFA overnight to ensure proper tissue fixation and preserve integrity. The fixed brains were then sectioned into coronal sections with a thickness of 50 µm using a vibratome.

5.5. ELECTROPHYSIOLOGY

5.5.1. ACC slice preparation

The mice were sacrificed for electrophysiological recordings 1-4 days following the behavioural session without memory recall. The mice were anaesthetised with isoflurane and intercardiacally perfused with ice-cold (4°C) artificial cerebrospinal fluid (aCSF) cutting solution (table 2.2). Following the perfusion, the mice were decapitated, the heads were immersed in the same ice-cold aCSF and the brains were removed rapidly. The posterior part of the brain without cerebellum was then glued to the cutting surface of the vibrating tissue slicer (Vibratome 3000 Plus, Sectioning Systems), with the dorsal side facing the blade. Coronal slices of 300 µm were cut and gently transferred using a pasteur pipette with a custom made open end into an incubating chamber filled with aCSF resting solution. Following 40 min of incubation at 37°C slices were transferred to a second incubation chamber containing aCSF recording solution (table 2.2). Then the slices were cooling down to room temperature (~ 23℃) for another 15 minutes. Brain slices were allowed to recover for at least one hour before electrophysiology recordings.

The mice were between 18 to 20 weeks old for electrophysiology experiments.

All the solutions were saturated with carbogen (95% O_2 with 5% CO_2) and adjusted the pH to 7.32~ 7.40 with HCl or NaOH. The osmolarity was adjusted to between 300~ 310 mOsm.

Ingredients	**Cutting solution (mM)**	**Resting solution (mM)**	**aCSF recording solution (mM)**
NaCl	-	124	124
NMDG	93	-	-
KCl	2.5	2.5	2.5
$NaH_2PO_4 \cdot H_20$	1.25	1.25	1.25
$NaHCO_3$	30	24	24
HEPES	20	5	5
D-Glucose	35	12.5	12.5
Thiourea	2	-	-

Na-ascorbate	5	1	-
Na-pyruvate	3	4	-
$CaCl_2 \cdot 2H_2O$	0.5	2	2
$MgSO_4 \cdot 7H_2O$	10	2	2

Table 2.2 Ingredients of extracellular solutions

5.5.2. Whole-cell recordings and pharmacology

Whole-cell recordings were performed using an upright microscope (Zeiss Examiner), a 63X/1.0 NA water immersion objective (Zeiss), and an IR-Dodt contrast system. An Evolve 512 EMCCD Camera (Photometrics) and automatic manipulators (Scientifica) were used. For whole-cell patch-clamp recordings, we used borosilicate glass capillaries (Harvard Instruments, GC150F-7.5) pulled by a PC-100 puller (NARISHIGE Group). The pipettes were filled with 10μl intracellular solutions, and the open-tip resistance ranged from 5 to 7 MΩ. The recording chamber of the electrophysiology setup was perfused with oxygenated aCSF, and the temperature was monitored and kept around 32℃ during the whole experiment.

The intracellular solution contained (in mM): 135 K-gluconate, 4 KCl, 10 HEPES, 10 Phosphocreatine-Na, 0.3 Na-GTP, 4 Mg-ATP and 0.3% Biocytin (Reference: 90055, Biotium). The osmolarity was adjusted to 290 mOsm, and the pH was set to be 7.30 with KOH. The intracellular solution was stored at -20℃ and kept on ice before use.

Data were collected in the bridge mode by an amplifier (Dagan, BVA-700C), low-pass filtered at 3k Hz, sampled at 20 kHz, and using AxoGraph X (version 1.7.6) software. Bridge resistance and capacitance were compensated and monitored during the recording period. Cells were discarded if the resting membrane potential was depolarized above -50 mV or applied bridge balance larger than 10 MΩ. While holding the cell's membrane potential at -70 mV, the electrophysiological properties were measured in the current-clamp mode.

To measure the intrinsic properties, an AMPA receptor antagonist (NBQX disodium salt, TOCRIS; concentration: 3uM in aCSF), a NMDA receptor antagonist (DL-AP5, TOCRIS; 50uM), and a $GABA_A$ receptor antagonist (SR 95531 hydrobromide, TOCRIS; 10uM) were bath-applied during the recording.

5.6. IMMUNOHISTOFLUORESCENCE AND IMAGING

5.6.1. Immunohistofluorescence staining for cFos and cell nucleus

Before carrying out the immunohistofluorescence, the coronal sections of brain taken and fixed were washed three times for 10 minutes with PB at 0.1 M to eliminate the residual PFA. Then the sections were incubated for 2 hours at room temperature in a blocking solution containing PB, 0.3% Triton X-100 and 5% BSA. It serves to reduce background noise and minimize non-specific binding of antibodies. After blocking, the sections were incubated with the primary antibody, cFos (9F6) Rabbit mAb #2250, a solution of rabbit polyclonal antibodies against cFos protein (1:1000 dilution) in the blocking solution overnight at room temperature protected from light. Following this, the sections were washed three times for 10 minutes with 0.1M PB, then incubated with a solution of secondary goat anti-rabbit Alexa 647 antibody labeled with the fluorophore Cy3 (dilution 1: 1000) for 2 hours at room temperature protected from light. The sections were then washed again three times for 10 minutes with 0.1M PB. To label the cell nuclei, the sections were incubated with DAPI (1:10000 dilution), for 10 minutes at room temperature, protected from light. Finally, sections were washed a final time with 0.1M PB and mounted on microscope slides using antifading mounting medium to preserve fluorescence.

5.6.2. Images acquisition and analysis

Biocytin filling and morphological reconstruction

After whole-cell patch-clamp recordings, the brain slices containing the Biocytin-filled (Biotium, reference: 90055) neurons were fixed in 4% PFA for two hours, then transferred to 0.1 M PBS solution at 4℃ for longer preservation. Brain slices were washed three times with 0.1 M PBS and then submerged for two hours at room temperature with a 0.7% Triton X-100 (EUROMEDEX). Slices were incubated with a solution that contained Streptavidin-Alexa 647/555 (1:1000, Thermo Fisher, Reference: S21318) and 0.3% Triton X-100 for three hours. The brain slices were washed with 0.1 M PBS three times and incubated with DAPI (1:10000, Sigma-Aldrich) for five minutes. All the brain slices were mounted with Mowiol-Dabco on microscopes slides with cover glasses.

The distance from the pia (middle line) was also measured to define the location of the layers in the ACC. Neurons in layer 2/3 of ACC were defined as 100-300 um from the pia surface,

while layer 5 tick-tufted pyramidal neurons were 350-650 um to the pia surface. Only post hoc identified layer 5 tick-tufted pyramidal neurons were included for analysis. The morphology of soma and dendrites were reconstructed with Neurolucida.

5.6.2.1. Confocal Imaging

Fluorescence images were then acquired using an SP8-MP confocal microscope equipped with Leica x20 oil immersion optics from the Bordeaux Imaging Center (BIC). Laser power and gain power remained the same for all slices. And the laser excitation wavelengths were determined as follows: 555 nm for mCherry, 503 nm for Green Lantern, 683 for Cy5 and 405 nm for DAPI. Once the images were acquired, they were imported into the specialized image analysis software IMARIS (version 10.0). Anterior Cingulate Cortex (ACC) areas of interest were manually delineated using the Fiji Image Analysis Tool (version 2.9.0), and their areas were extracted. A median filter with an intensity of 3*3*3 was applied equally to all channels for image processing. cFos-positive and mCherry or Green Lantern-positive neurons were identified and quantified based on their fluorescence intensity and size. Then we created a new channel to co-localize cFos-positive and mCherry/Green Lantern-positive neurons. Information such as the position of neurons on the x-axis, the number of neurons detected in each category, the thickness of layer 1 of the cortex and the maximum thickness of the ACC were extracted. All imaging and analyzes were performed blind to the experimental conditions.

5.6.2.2. Slide scanners

The high-resolution fluorescence images were then acquired using a Hamamatsu NANOZOOMER 2.0HT slide scanner from the Bordeaux Imaging Center (BIC). Once the images were acquired, they were imported into the Fiji analysis software (version 2.9.0) and the hippocampal areas of interest were delineated manually. Then the number of cFos positive neurons was extracted. A Gaussian filter was first applied and then using the “Find Maxima” and “3D Objects Counter” function of the number of positive cFos neurons detected and the area of the region of interest has been extracted. All imaging and analyzes were performed blind to the experimental conditions.

5.7. BIOCHEMICAL ANALYSIS

5.7.1. Western Blot

5.7.1.1. Protocol

Protein amounts of brain homogenates were determined by the Bradford's protein assay and normalized to 5-20 µg of protein per sample. Twelve percent NuPAGE Bis-Tris polyacrylamide gels (Invitrogen, France) were used to run the electrophoresis (80 min, 120V). After size separation within the gel, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Polyscreen® membrane, Perkin Elmer, France). Membranes were blocked with a solution containing 0.1% Tween 20 and 200 mM Tris buffered solution (TTBS) complemented with 5 % non-fat dry milk during 30 min and incubated with primary antibody, at 4°C overnight under gentle agitation. Incubation with the secondary fluorescent-conjugated antibody was performed during 1 h at room temperature. After 3 washes with TTBS and one with PBS the membrane was scanned using a Licor Aerius automated infrared imaging system according to manufacturer's instructions. Quantification based on band intensity was done with the software provided with the imaging system.

Because of the low amount of sample, the membrane was reused two times. Antibody deshybridation was performed by incubating the membrane in a stripping buffer (0.2 M glycine, 0.1 % SDS, 1 % Tween 20, adjusted pH 2.2) during 5-10 min. After several washes with TTBS, the membrane was scanned to control the efficiency of the stripping buffer. After this control, the membrane was incubated with TTBS containing 5 % non-fat dry milk and then incubated with another antibody in the same conditions as described before.

5.7.1.2 Antibodies

Different primary antibodies were used: 6E10 (BioLegend, 1 µg/ml), Anti-GFAP (TermoFisher, 10 µg/ml), anti-Actin (Sigma Aldrich, 0.8 µg/ml), anti-Tau (TermoFisher, 0.5 µg/ml). The adequate secondary antibodies were used: Goat anti-Rabbit (IR Dye ® 800CW, LI-COR) or Goat anti-Mouse (IR Dye ® 680 RD, LI-COR).

5.8. DETERMINATION OF BRAIN AB_{1-42} BY ELISA ASSAY

Human Aβ1-42 was quantified in the same amount of ACC extract from 3xTG mice and WT littermates by ELISA (BetaMark Covance, USA). According to the manufacturer, this kit specifically detects soluble forms of $Aβ_{1-42}$ with negligible cross-reactivity to $Aβ_{1-40}$. Aβ concentration in samples was read in a 96-well plate against a standard curve (0-250 pg/ml). Chemiluminescence signals were detected using a microplate reader.

5.9. STATISTICAL ANALYSIS

The data obtained during the different experimental stages were subjected to statistical analysis to determine the significance of the results and to assess the differences between the groups. Statistical analyzes were performed using appropriate statistical software GraphPad Prism (Version 9.5.1). First, data were checked for normality and homogeneity of variance using tests such as Shapiro-Wilk and Ficher respectively. If the data were normally distributed and had homogeneous variance, parametric tests were used to analyze differences between groups. Otherwise, non-parametric tests were applied. For comparisons between two groups, the Student's t-test or the Mann-Whitney test was used, depending on the nature of the data. For comparisons between more than two groups, an analysis of variance (ANOVA) was performed, followed by post-hoc Šídák tests to identify specific differences between groups. Results are presented as means ± S.E.M. or medians and interquartile ranges, depending on the distribution of the data. Differences were considered statistically significant when the p-value was less than 0.05. Graphs and tables have been created to illustrate the results in a clear and understandable way.

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