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METABOLIC CROSS-TALK BETWEEN ASTROCYTES AND NEURONS: IMPLICATIONS FOR ALZHEIMER'S DISEASE

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1 Introduction

1.1 Alzheimer’s disease

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disorders, posing a significant global health challenge. AD is the most common cause of dementia, accounting for a significant portion of dementia cases. Dementia is an umbrella term used to describe a range of cognitive impairments that interfere with an individual's ability to think, remember, and perform daily activities. The World Health Organization reports that dementia impacts around 55 million individuals globally, with 60–80% of these individuals receiving a diagnosis of AD. Most AD cases are considered sporadic, emerging later in life (between 60 and 65 years), and are influenced by intricate interplays between genetics and environmental factors [1].

Named after Alois Alzheimer, the German psychiatrist who first identified and described the condition in 1906, this disease primarily affects the brain, causing a gradual decline in cognitive abilities and memory function [2]. As a form of dementia, AD disrupts various cognitive processes, leading to difficulties in thinking, reasoning, and daily functioning. Over time, the condition profoundly impacts on individual's independence, quality of life, and relationships.

AD is generally classified into two main types based on age of onset: early-onset Alzheimer's disease and late-onset Alzheimer's disease [3]. The Early-Onset Alzheimer's Disease (EOAD) occurs before the age of 65. It is relatively rare, accounting for about 5-10% of all Alzheimer's cases. EOAD can be caused by genetic mutations inherited from one's parents; three genes are associated with this type of AD: amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2), mutations in any of these genes can lead to the early development of the disease [4]. The most frequent form of AD is the Late-Onset Alzheimer's

Disease (LOAD) and typically occurs after the age of 65. It is believed to result from a complex interaction of genetic, environmental, and lifestyle factors. APOE ε4, a variant of the apolipoprotein E gene, a protein involved in the metabolism of lipids in the body, including cholesterol, is the most well-known genetic risk factor for LOAD. However, having the APOE ε4 allele does not guarantee the development of the disease, and many individuals without this allele still develop AD [5]. There are also some rare genetic variations that can cause different forms of the disease. The Familial Alzheimer's Disease (FAD) refers to cases where AD runs in families and is caused by inherited genetic mutations. FAD is relatively rare and is often associated with EOAD. Mutations in genes such as APP, PSEN1, and PSEN2 can lead to the familial form of the disease [6]. Sporadic Alzheimer's Disease (SAD) occurs without a clear family history. It usually develops later in life, typically after the age of 65, while genetic factors may still play a role, the exact cause of SAD is less clear and is likely influenced by a combination of genetic, environmental, and lifestyle factors [6]. It's important to note that while these categories provide a general framework for understanding the different types of AD, the condition itself is complex and can vary widely in its presentation and progression.

AD is characterized by specific pathological hallmarks that contribute to the cognitive decline and neurological symptoms associated with the condition [7]. The two primary hallmarks of AD are amyloid plaques and neurofibrillary tangles. Amyloid plaques are abnormal accumulations of protein aggregates mostly composed of amyloid-beta (Aβ) peptides. Aβ peptides are fragments of APP, which is normally processed through two main pathways: the non-amyloidogenic and amyloidogenic pathways [8] (Fig. 1). In the non-amyloidogenic pathway, APP is cleaved by an enzyme known as alpha-secretase, this cleavage occurs within the Aβ region of APP, preventing the formation of Aβ peptides; it

produces soluble APP-alpha (sAPP-alpha), which is released into the extracellular space. This pathway is considered protective because it reduces the production of Aβ peptides, which are associated with neurotoxicity [9]. The amyloidogenic pathway involves different enzymes and results in the generation of Aβ peptides. Beta-secretase (BACE1) cleaves APP at the N-terminus of the Aβ region, producing soluble APP-beta (sAPP-beta) and a membrane-bound C-terminal fragment (C99). C99, in turn, is cleaved by the gamma-secretase complex [10]. This cleavage results in the generation of various Aβ peptides, including Aβ1-40 and Aβ1-42. Aβ peptides, especially Aβ1-42, have a propensity to aggregate and form insoluble fibrils. These aggregates accumulate over time and eventually become amyloid plaques, which can disrupt synaptic function, trigger neuroinflammation, and contribute to the neurodegeneration seen in AD [11].

The initial investigations into the impact of bioenergetics on various APP processing pathways revealed that disturbances in cellular energy resulting from uncoupling agents cause the accumulation of APP within the Golgi apparatus. Additionally, Heriques and colleagues [12] demonstrated that a deficiency in glucose availability in COS cells (fibroblast-like cell lines derived from monkey kidney tissue) results in a reduction in the levels of secreted sAPPα in addition to a glycolysis inhibitor, 2-deoxy-D-glucose (2DG), suggesting that changes in glucose supply can influence APP processing. BACE1 transcription appears to be influenced by the same factors that regulate mitochondrial biogenesis and bioenergetics [13]. This suggests a potential link between cellular energy status and the transcriptional regulation of BACE1. Dysregulation of BACE1 expression can have significant implications for APP processing and the production of Aβ peptides. The gamma receptor, which is activated by peroxisome proliferator (PPARγ), plays a role in regulating the transcription of both BACE1 and peroxisome proliferator-activated receptor

coactivator 1-alpha (PGC1α), which is known for its involvement in mitochondrial biogenesis [13]. When PGC1α is overexpressed in neuroblastoma N2a cells, it leads to a decrease in the secretion of Aβ and an increase in the secretion of sAPPα [14]. While insoluble Aβ plaques were initially the focus of Alzheimer's research, there is growing recognition that soluble Aβ oligomers, especially small, soluble forms, are the primary drivers of neurodegeneration and cognitive decline in the context of AD. Soluble Aβ oligomers are considered highly pathogenic in AD for several key reasons:1) are known to be neurotoxic, they can disrupt synaptic function, impair neuronal communication, and induce oxidative stress, leading to neuronal injury and cell death; 2) they have been shown to disrupt synaptic plasticity, which is essential for learning and memory, this synaptic dysfunction is believed to underlie the cognitive deficits seen in AD; 3) they can trigger an inflammatory response in the brain, leading to the activation of microglia and astrocytes, which is detrimental to neuronal health; 4) they induce oxidative stress within the brain, which can damage cellular components, including lipids, proteins, and DNA, further exacerbating neuronal dysfunction and cell death; 5) they induce the hyperphosphorylation of Tau protein and lead to neuritic degeneration [15,16]. Accordingly, one hypothesis in AD research is that amyloid plaques could serve as a reservoir from which soluble Aβ oligomers can diffuse [17]. This concept is often referred to as the "amyloid cascade hypothesis", which suggests that the aggregation of Aβ into plaques is just one aspect of the disease process.

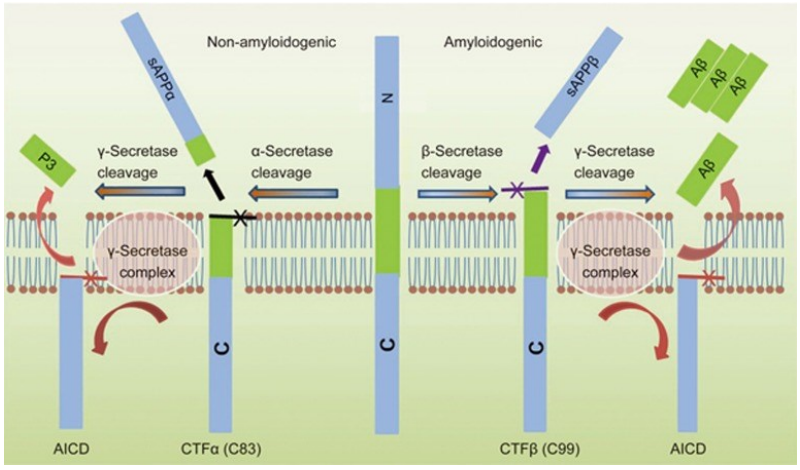


Figure 1: The non-amyloidogenic and amyloidogenic pathways of APP [10].

The other important hallmark of AD is the presence of neurofibrillary tangles (NFTs) composed of hyperphosphorylated Tau protein (pTau). Tau protein plays a critical role in the pathological development of AD. Nevertheless, it's important to note that alterations in the Tau protein gene by themselves are not sufficient to trigger the onset of AD [18]. In contrast, genetic alterations related to Aβ are implicated as direct contributors to the condition, as just mentioned.

Tau protein is essential for maintaining the structural integrity of neurons by stabilizing microtubules, which are responsible for transporting nutrients and other essential molecules within the cell [19].

Indeed, Tau is a family of six different protein isoforms that result from the alternative splicing of a single gene known as the "MAPT" (Microtubule-Associated Protein Tau). These six forms of Tau differ based on the presence of zero, one, or two inserts of 29 amino acids near the N-terminus (N1 and N2) and the presence of 3 or 4 tubulin binding sites near the C-terminus (3R or 4R). Tau isoforms are expressed in different ratios in the human brain, and their proportions can change under various conditions or in neurodegenerative diseases

like AD and other tauopathies [20]. The presence or absence of these inserts and the number of microtubule-binding repeats influence Tau's ability to interact with microtubules and other cellular structures, which can have significant implications for normal neuronal function and disease pathology. Tau isoforms with four microtubule-binding repeats (4R) are more prone to pathological hyperphosphorylation, an excessive addition of phosphate groups compared to their usual state, and aggregation in conditions like AD [21].

In the context of AD, this altered phosphorylation causes Tau proteins to lose its ability to bind to microtubules. As a result, microtubules destabilize, leading to structural abnormalities in neurons and impaired intracellular transport. pTau has a greater tendency to aggregate and form paired helical filaments (PHFs), which are a major structural component of neurofibrillary tangles (NFTs). These tangles disrupt the transport system and overall cellular function, leading to neuronal damage and eventual cell death [21]. The hyperphosphorylation of Tau in neurodegenerative diseases like AD is primarily due to conformational alterations in Tau itself, which make it a more permissive substrate for phosphorylation by kinases, enzymes that add phosphate groups to proteins. These conformational changes can be driven by various factors, including genetic mutations, environmental influences, and the presence of pathological proteins [22]. Hyperphosphorylation of Tau is primarily driven by the abnormal activation of protein kinases, which are often overactive or dysregulated in neurodegenerative diseases. These aberrantly activated kinases phosphorylate Tau at multiple sites, leading to hyperphosphorylation. pTau may also be less permissive for dephosphorylation by protein phosphatases, which are enzymes responsible for removing phosphate groups from proteins. The conformational changes in pTau may hinder the access of phosphatases to their target sites, making it challenging to reverse the hyperphosphorylation. The interplay between

Tau's altered conformation, abnormal kinase activity, and reduced dephosphorylation contributes to the vicious cycle of Tau hyperphosphorylation and aggregation in neurodegenerative diseases [23,24].

In AD, pTau levels in the brain are typically elevated compared to healthy individuals. pTau constitutes two pools: the Neurofibrillary Tangle-Bound pTau and the non-fibrillar cytosolic pTau. NFT-Bound pTau is characterized by aggregated pTau, which become part of NFTs or other Tau aggregates within the neurons, they are composed of hyperphosphorylated Tau protein that has adopted a fibrillar, insoluble conformation. Non-fibrillar cytosolic pTau refers to phosphorylated Tau that remains in a soluble, non-aggregated form within the cytoplasm of neurons. This pool of pTau may include Tau molecules that are hyperphosphorylated but have not yet aggregated into NFTs. It’s a form of Tau that has undergone reversible phosphorylation and is involved in normal cellular processes related to microtubule dynamics and intracellular signaling [25] (Fig. 2).

In summary, pTau and Aβ are central players in the pathogenesis of AD; while the exact sequence of events and causal relationship between Aβ and pTau in AD is still under investigation, there is strong evidence to suggest that these two pathological features are interconnected and contribute to the progression of the disease.

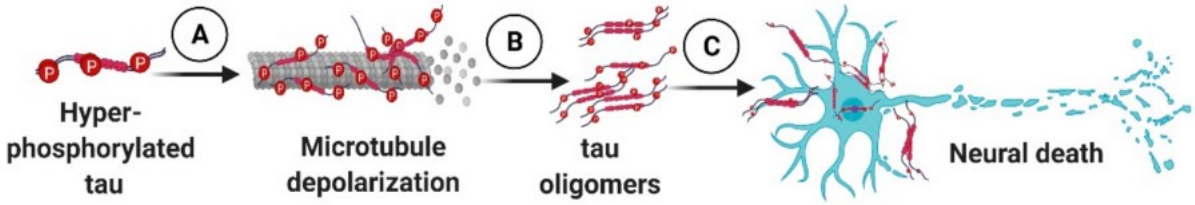


Figure 2: The hyperphosphorylation of tau protein (A) leads to microtubule depolarization (B), tau oligomer aggregation, neurofibrillary tangle formation (C), and subsequent neuronal cell death with the release of tau oligomers into the extracellular matrix [26].

1.2 The complicated system of interactions between energy metabolism, calcium homeostasis and oxidative stress in AD

In addition to the accumulation of Aβ and pTau, recognized as primary causes of the disease, other factors contribute to AD, such as the loss of neurons, degeneration of synapses, increased presence of reactive astrocytes, changes in the glial phenotype, and oxidative stress in microglia. These alterations in the brain associated with AD arise from an intricate interplay of various factors, encompassing age, genetics, chronic inflammation, disruptions in energy metabolism and mitochondrial dysfunctions.

The complex network of interactions between energy metabolism, calcium homeostasis, and oxidative stress in AD is a complex and multifaceted process that plays a significant role in the pathogenesis of the disease (Fig. 3). Among the processes increasingly recognized as pivotal in driving the onset and advancement of the disease, mitochondrial dysfunction has recently emerged as the primary instigator of a cascade of events ending in neuronal degeneration [27]. In AD, there are several key points linked to mitochondrial dysfunction: the increased Reactive Oxygen Species (ROS) production, the dysfunction of energy metabolism and the induction of apoptotic events. These processes are also linked to another

important cellular mechanism, the dyshomeostasis of calcium, which involves the equilibrium of intracellular calcium levels. Mitochondria are involved in maintaining the balance of intracellular calcium levels, but in AD, this balance is disrupted, leading to an excessive influx of calcium into mitochondria. This calcium overload further compromises mitochondrial function and adds to cellular stress [28]. Calcium regulation depends on ATP-dependent enzymes which can be influenced by ROS through conformational changes [29]. Heightened calcium signaling can potentially serve as a catalyst for the generation of reactive oxygen species (ROS). This, in turn, could impair mitochondrial function and disrupt energy equilibrium, initiating a detrimental cycle that exacerbates neurodegenerative processes.

1.2.1 Energy metabolism

Energy metabolism in the brain is a highly dynamic and tightly regulated process that ensures the brain's energy demands are met for its various functions, including cognition, memory, and neural signaling [30]. The substantial energy requirements of neurons make them highly sensitive to insufficient energy supply, and any disruption can result in a range of diseases. As a result, the functioning and physiology of the brain are closely linked to glucose. The metabolic processing of glucose in the brain is subject to the influence of various factors, including the specific needs of brain cells. This metabolic process primarily encompasses the following routes: 1) Glycolysis can convert glucose into either pyruvate or lactate, which subsequently enters oxidative phosphorylation (OxPhos); 2) Glucose can also be channeled into glycogenesis, resulting in the creation of glycogen deposits in astrocytes, serving as an energy reserve; 3) Glucose can also be used to generate 5-carbon sugars and nicotinamide adenine dinucleotide phosphate (NADPH) through the pentose phosphate pathway (PPP). This pathway potentially provides a defense mechanism against oxidative stress [31].

Different types of brain cells may exhibit preferences for these pathways based on their distinct functions and energy requirements. This versatility in glucose metabolism is essential for the brain's ability to adapt to varying energy demands and oxidative stress levels.

Glucose metabolism in AD context is compromised [27]. Indeed, in AD, there is evidence of reduced glucose utilization and impaired glycolysis in affected brain regions. This means that the brain is less efficient at using glucose to produce energy (ATP) through normal metabolic pathways. In literature it is known that reduced glucose utilization is linked to ROS production, frequently due to oxidative modification of specific enzymes involved in glucose metabolism. A vicious cycle can develop, where oxidative stress leads to enzyme modification and dysfunction, which in turn results in reduced glucose metabolism and energy deficits. This cycle can perpetuate oxidative damage and contribute to disease progression.

Oxidative stress is a major contributor to mitochondrial DNA impairment, which consequently leads to inefficient energy production. From this it is important to underline the fact that mitochondrial dysfunction is closely correlated with insulin resistance.

Insulin resistance is a key feature of type 2 diabetes, which is a condition in which the body's cells do not respond properly to the hormone insulin [32]. In brain, insulin resistance refers to a condition in which brain cells, particularly neurons, do not respond effectively to the hormone insulin. While insulin is primarily associated with regulating blood sugar levels in the body, it also plays important roles in the brain, including regulating glucose uptake, energy metabolism, and synaptic plasticity [33].

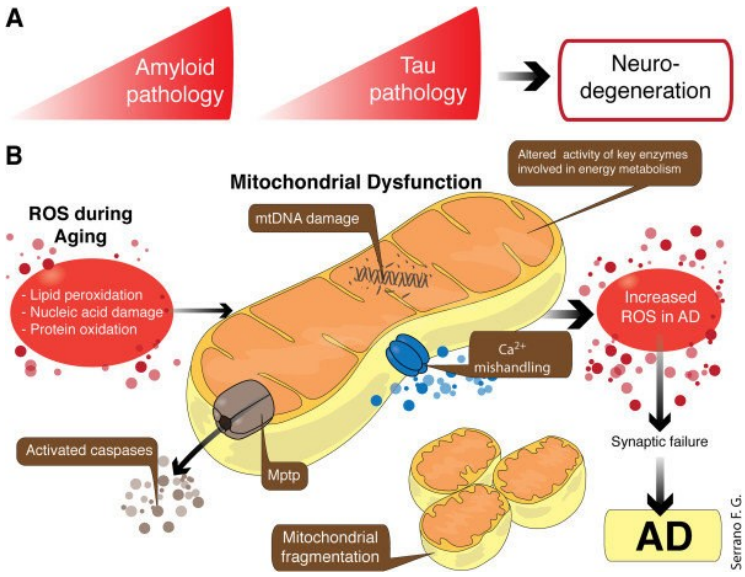


Figure 3: Hallmarks of AD, progression of the disease and mitochondrial dysfunction [34].

1.2.2 Advanced Glycation End-products (AGEs)

The phenomenon of glycation, or non-enzymatic glycosylation, plays a significant role in the intricate connection between diabetes and AD. This process involves the binding of sugars, most notably glucose, to proteins, leading to the formation of Advanced Glycation End-products (AGEs). In the context of diabetes, where blood sugar levels are often elevated over extended periods, glycation is accelerated, resulting in higher accumulation of AGEs [35]. The increase of AGEs generation occurs also in pathological conditions, such as inflammatory states, oxidative stress, or hyperglycemia [36].

In the case of AD, the role of AGEs is increasingly recognized. AGEs can promote oxidative stress, inflammation, and the aggregation of pTau and Aβ, they also impair synaptic function and contribute to neurodegeneration [37].

AGEs can bind to a cell surface receptor known as RAGE, which stands for the "Receptor for Advanced Glycation End-Products", a member of the immunoglobulin superfamily of cell surface molecules, which is expressed in different cell types (i.e., neurons, microglia,

hepatocytes, hepatic stellate cells (HSC), cardiomyocytes, fibroblasts and endothelial cells (EC)) [38]. When AGEs interact with RAGE, they initiate signaling pathways within cells. This activation of RAGE can lead to several cellular responses. Activation of RAGE by AGEs triggers intracellular signaling pathways lead to the release of pro-inflammatory factors and molecules, including Tumor Necrosis Factor α (TNFα) and Nuclear Factor kappa B (NF-kB). This promotes inflammation which is linked to the increased production of ROS [39]. RAGE can bind to Aβ, this interaction is one of the mechanisms by which Aβ may exert its harmful effects in the brain in the context of AD [40].

In 2009, Takeuchi and colleagues identified several cytotoxic classes of AGEs, named toxic AGEs (TAGE), which contribute to cytotoxic effects in various cell types [41]. These specific molecules are generated as intermediates in response to aberrant glucose and fructose metabolism, particularly when excess glyceraldehyde (GA) is present in the cellular environment [42].

In health conditions, GA is enzymatically converted into glyceraldehyde-3 phosphate (GA3P) by the action of glyceraldehyde-3 phosphate dehydrogenase (GAPDH), effectively maintaining low levels of GA. Concomitant with the reduction in GAPDH activity, there is a decrease in the metabolism of GA3P, resulting in an intracellular accumulation. Consequently, the processing of GA3P shifts to an alternative pathway, leading to an elevated level of GA, thereby increasing the production of glyceraldehyde-derived AGEs (Glycer-AGEs) [43]. In AD affected brain, GAPDH is susceptible to substantial alterations, (including oxidative and post-translational modifications), with the consequence changing in its structural integrity and functional activity.

It has been observed in literature that exposure SH-SY5Y neuroblastoma to GA cells leads to an elevated production of AGEs [44]. This increase in AGEs subsequently disrupts the

regulation of AD biomarkers and ultimately results in cell death. These observations underscore the potential involvement of AGEs and GA in the complex pathophysiology of AD [45].

1.2.3 Calcium homeostasis

Calcium plays a fundamental role in numerous cellular processes, including neurotransmitter transmission, synaptic contact, cell proliferation, apoptosis, and acting as a versatile and ubiquitous intracellular messenger. Calcium homeostasis is intricately linked to the pathogenesis of AD. Disruptions in calcium signaling and regulation can contribute to neuronal dysfunction and neurodegeneration [46]. For these reason receptor, ion channels and transporters play a fundamental role in the maintenance of calcium concentrations which are different in cytoplasm (~100 nM), extracellular environment (~1.2 mM) and inside intracellular organelles, for example endoplasmic reticulum (ER) and lysosomes (0.5-1 mM) [47]. ER serves as a major intracellular calcium reservoir. The Sarco/Endoplasmic Reticulum Calcium ATPase (SERCA) is an enzyme responsible for actively transporting calcium ions from the cytoplasm back into the lumen of the ER and the sarcoplasmic reticulum (SR) in muscle cells [47]. SERCA activity is strictly regulated by oxidative phosphorylation, ROS production, calcium levels [48] and also by the protein presenilin [49].

Calcium release from ER and SR is mediated by two ion channels, located on its membrane: the Inositol 1,4,5-Trisphosphate Receptor (I3PR) and the Ryanodine Receptor (RyR). IP3R is activated in response to the binding of inositol 1,4,5-trisphosphate (IP3), a second messenger produced when certain cell surface receptors are stimulated. RYR is involved in the release of calcium ions during muscle contraction, especially in skeletal and cardiac muscle cells. RYR can be activated by calcium-induced calcium release (CICR), where a

small influx of calcium through other channels triggers a larger release of calcium from the SR, leading to muscle contraction.

The regulation of intracellular calcium levels is also mediated by the Store-Operated Calcium Entry (SOCE); this mechanism plays a crucial role in various cellular processes, including signal transduction, gene expression, and immune responses. When intracellular calcium stores are depleted, SOCE replenishes the calcium stores and maintains cellular calcium homeostasis [50].

As previously mentioned, calcium dysregulation is implicated in the pathophysiology of AD. Abnormal calcium signaling can disrupt synaptic function, promote neuronal injury, and impact the processing APP. Presenilin plays a critical role in the γ-secretase complex, which cleaves APP to produce Aβ peptides. Mutations in presenilin genes alter γ-secretase activity, leading to increased production of $A\beta_{42}$, a particularly toxic form of Aβ. There is evidence suggesting that calcium ions can modulate γ-secretase activity, potentially by interacting with presenilin. Calcium ions may influence the cleavage of APP by γ-secretase and, consequently, the production of Aβ peptides. Dysregulated calcium signaling may contribute to increased Aβ production in AD [51].

Mitochondria is highly influenced by calcium release from ER. The increased levels of calcium within mitochondria can activate enzymes like the α-KetoGlutarate DeHydrogenase Complex and the Pyruvate DeHydrogenase Complex (PDHC). These enzyme complexes are involved in key metabolic pathways, such as the citric acid cycle (Krebs cycle) and pyruvate decarboxylation, which contribute to the generation of ATP. Activation of these complexes leads to an increase in the production of reduced nicotinamide adenine dinucleotide (NADH) within the mitochondria, with the consequent saturation of the mitochondrial respiratory chain. This saturation occurs because NADH donates electrons to the respiratory chain,

which can potentially overwhelm the capacity of the chain to process these electrons. When the respiratory chain becomes saturated, there is an increased likelihood of electron leakage, leading to the generation of ROS [52]. This intricate interplay between calcium signaling and mitochondrial function is crucial for cellular energy production and can also have implications for oxidative stress and cellular health.

1.2.4 Oxidative stress

Oxidative stress is a biological condition that arises when there is an imbalance between the production of ROS, such as free radicals, and the body's ability to neutralize and detoxify them using antioxidants. ROS production is a natural and essential part of cellular metabolism in aerobic organisms, they are highly reactive molecules containing oxygen, such as superoxide radicals (O_2•−), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH). ROS are formed as byproducts during various cellular processes, including mitochondrial respiration, enzymatic reactions, and immune system responses. While ROS serve important roles in cellular signaling, defense against pathogens, and regulation of certain physiological processes, excessive ROS production can be detrimental [53].

The brain is particularly vulnerable to oxidative stress due to its high metabolic rate, relatively low levels of antioxidant defenses compared to other organs, and its high content of easily oxidizable lipids. The brain exhibits heightened susceptibility to ROS production for various significant factors. These include its abundance of easily peroxidizable polyunsaturated fatty acids, elevated levels of redox-active transition metal ions such as iron (Fe) and copper (Cu), the involvement of dopamine and glutamate oxidation in synaptic transmission, and the presence of a substantial number of resident immune cells, all of which can serve as pro-oxidants [54]. Mitochondria is one of the primary sources of ROS in brain.

In the electron transport chain (ETC), electrons are transferred through a series of protein complexes. Occasionally, some electrons "leak" from the chain, especially at complex I and complex III, which can interact with oxygen molecules, leading to the formation of O_2•−. The superoxide radicals are highly reactive and can be converted into H_2O_2 through a process known as disproportionation [55]. This conversion is facilitated by the enzyme superoxide dismutase (SOD), which helps to detoxify superoxide radicals. To counteract ROS production, mitochondria have their own antioxidant defense systems. These include enzymes like manganese superoxide dismutase (MnSOD), which helps convert superoxide radicals into less harmful molecules, and other antioxidants like glutathione [54]. Oxidative stress is believed to play a significant role in the development and progression of AD through several mechanisms. In brain tissues of patients with AD, oxidative stress can lead to the oxidative modification of key glycolytic enzymes, like GAPDH, Phosphoglycerate mutase 1 (PGM1), Triosephosphate isomerase (TPI), which can have significant implications for brain metabolism and function [27]. Excessive ROS production can lead to oxidative damage to cellular components, including proteins, lipids, and DNA, which can impair cellular function and contribute to neurodegeneration; more over Aβ peptides, particularly the Aβ42 form, generate ROS and induce oxidative stress. Chronic inflammation in the brain, known as neuroinflammation, is a characteristic feature of AD. Inflammatory processes can stimulate the release of ROS and reactive nitrogen species (RNS), amplifying oxidative stress and causing further damage to neurons. Mitochondrial function is impaired due to oxidative stress, leading to decreased energy production and increased ROS levels, thus leading to a vicious circle of further oxidative stress which is also linked to calcium dyshomeostasis in mitochondria as mentioned previously [56].

1.2.5 Inflammation

In AD inflammation, specifically neuroinflammation, plays a fundamental yet intricate role. It represents a dynamic response of the brain's immune system to the pathological changes characteristic of AD, which involve various immune cells and inflammatory molecules. Microglia, the brain's resident immune cells, become activated in response to the presence of Aβ plaques and other pathological alterations [57]. While microglia can help clear debris, their prolonged activation can also release proinflammatory and toxic products, including ROS, nitric oxide, and cytokines, contributing to neuronal damage. The chronic activation of microglia can also impair their ability to carry out beneficial functions, such as supporting synaptic maintenance and promoting tissue repair. The ongoing release of pro-inflammatory cytokines and neurotoxic compounds by microglia further intensifies neuroinflammation and contributes to the process of neurodegeneration, which in turn triggers the activation of additional microglia [58]. This vicious cycle perpetuates the inflammatory response and is implicated in the progressive deterioration of neural tissue [59]. Simultaneously, astrocytes, another type of glial cell, become reactive in AD. They play a multifaceted role in the disease, releasing inflammatory mediators and assisting in the maintenance of the blood-brain barrier. The dysfunction of this barrier is a hallmark of AD and can lead to further complications, underlining the complex and interconnected roles of these glial cells in the pathogenesis of the disease [60]. The role and activation of astrocytes will be discussed in subsequent chapters.

1.3 Astrocytes

Astrocytes are multifunctional star-shaped glial cells that play a pivotal role in maintaining the health and function of the central nervous system (CNS). Their significance has been recognized and explored ever since their histological identification by Ramon y Cajal and his contemporaries over a century ago. Initially, it was thought that astrocytes primarily provided structural support to neurons, but then, Ramon y Cajal's work revealed the morphological diversity among astrocytes, identifying nine different subtypes and giving rise to numerous theories regarding their crucial functions [61].

These cells are abundant in the brain and spinal cord, where they perform a wide array of functions critical for neuronal well-being. Astrocytes provide structural support to neurons by forming a network of processes that help maintain the physical integrity of neural tissue; they are crucial in the formation and maintenance of the blood-brain barrier (BBB), a protective interface that regulates the passage of substances between the bloodstream and brain tissue, preventing harmful substances from entering the brain [62]. Additionally, they are involved in the regulation of neurotransmitters, helping to clear and recycle neurotransmitters like glutamate, ensuring the precise termination of synaptic signaling and preventing excessive stimulation of neurons [63]. They play a role in maintaining ion balance in the extracellular space, helping to regulate the concentrations of ions such as potassium and calcium, which are vital for neuronal function. Astrocytes supply energy substrates, such as glucose and lactate, to neurons, ensuring that they have the necessary nutrients for their metabolic needs; they also have neuroprotective functions, including antioxidant defense and the removal of excess neurotransmitters, which help shield neurons from oxidative stress and excitotoxicity [64]. They are involved in synaptogenesis, contributing to the formation and maturation of synapses between neurons during brain

development and in response to learning and memory processes. Astrocytes participate in immune responses within the CNS. They can release pro-inflammatory or anti-inflammatory molecules and play a role in neuroinflammation, which can influence the outcome of neurological diseases and injuries [65]. Dysfunctional astrocytes are implicated in various neurological disorders, including neurodegenerative diseases like Alzheimer's and Parkinson's disease, as well as conditions like multiple sclerosis and epilepsy [66].

1.3.1 Reactive Astrocytes

In response to various forms of injury, disease, or pathological conditions, astrocytes became reactive. These specialized glial cells undergo substantial morphological and functional changes in response to neural insults which can have both positive and negative roles in the context of various neurological conditions, depending on the circumstances and the stage of the disease or injury [67] (Fig. 4). Here are some of the positive functions. Reactive astrocytes (RA) are essential for wound healing in the CNS, they can form a glial scar, which acts as a physical barrier, limiting the spread of damage and inflammation, and preventing further injury to healthy tissue [68]. Astrocytes release neurotrophic factors like brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) which promote neuronal survival, growth, and regeneration, helping to protect neurons from damage [69]. RA can produce and release antioxidants, which help counteract the harmful effects of oxidative stress and ROS during inflammation and injury [70]. Astrocytes are involved in maintaining optimal levels of neurotransmitters and ions in the extracellular space, contributing to synaptic plasticity and supporting normal neuronal function. In some cases, RA exhibit phagocytic activity, assisting in the clearance of cellular debris and damaged components from the CNS, which is crucial for tissue repair [71]. Below are some

of the common negative molecular changes associated with the activation of astrocytes. One of the hallmark features of RA is the upregulation of glial fibrillary acidic protein (GFAP), an intermediate filament protein, which leads to changes in the astrocyte's cytoskeleton, making them more rigid and supportive [72]. RA release pro-inflammatory cytokines and chemokines such as interleukin-1β (IL-1β), TNF-α, and interleukin-6 (IL-6). These molecules play a role in initiating and amplifying the inflammatory response within the CNS. They can also release neurotrophic factors such as brain-derived neurotrophic factor (BDNF). These factors may have both neuroprotective and neurotoxic effects depending on the context. RA produce ROS which contributes to oxidative stress in the CNS [73]. They may exhibit changes in intracellular calcium signaling, which can influence their interactions with neurons and other glial cells. Calcium signaling plays a crucial role in astrocyte functions [74]. They may alter the expression of ion channels, affecting their ability to regulate extracellular ion concentrations and neurotransmitter homeostasis [75]. In response to stressors, RA may experience mitochondrial dysfunction, which can contribute to their altered functions and production of ROS [76]. In certain neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, the chronic activation of astrocytes may exacerbate disease progression by promoting inflammation and impairing neuronal function.

1.3.2 Altered characterization of reactive astrocytes related to AD

Astrocytes play a complex and multifaceted role in AD. While these glial cells play important roles in supporting neuronal health and functioning, in the context of AD, their responses can become dysregulated and detrimental, contributing to disease progression. RA in AD brains often impact to chronic neuroinflammation, the release of pro-inflammatory cytokines and chemokines exacerbate neuronal damage and promote the recruitment of

immune cells into the brain [77]. As a part of their response to neuroinflammation or oxidative stress, astrocytes can produce ROS, which if increased, contribute to oxidative damage to neurons. Astrocytes may accumulate Aβ peptides, these "Aβ-associated astrocytes" can contribute to the amyloid plaque burden in the brain. Dysfunctional astrocytes may fail to properly regulate neurotransmitter levels, particularly glutamate, this can lead to excessive glutamate in the synaptic cleft, promoting excitotoxicity and neuronal death [78]. In AD, astrocytic dysfunction can lead to BBB breakdown, allowing harmful molecules and immune cells to enter the brain exacerbating inflammation and contributing to neurodegeneration. Astrocyte dysfunction can affect energy metabolism in the brain. Disrupted astrocytic support for neuronal energy needs can contribute to neuronal dysfunction and vulnerability in AD.

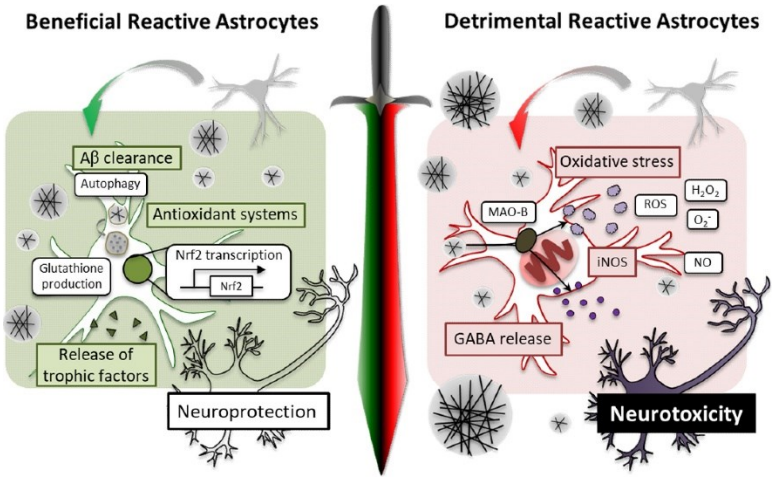


Figure 4: Beneficial and detrimental reactive astrocytes in AD [79].

1.3.2.1 Astrocytes in Amyloid and Tau protein metabolism in AD

The hypothesis regarding the etiology of AD suggests that the accumulation of Aβ plaques and the formation of neurofibrillary tangles of pTau in the brain activate an inflammatory response, which stimulates microglia, to release various inflammatory mediators. There is evidence suggesting a connection between astrocytes and amyloid peptides in AD. For example, astrocytes have been shown to play a role in degrading Aβ plaques through enzymatic cleavage [80]. Additionally, they are capable of internalizing Aβ1–42 both in in vitro and in vivo, as demonstrated in mouse models and human cell cultures [81]. RA and BACE1 activation are often linked, as demonstrated by Heneka and colleagues, who observed an accumulation of Aβ in transgenic mice with an overexpression of mutant APP [82]. Moreover, the release of IFN-γ and TNF in RA is linked to elevated levels of both BACE1 and APP, subsequently leading to an increase in Aβ production [83]. Astrocytes, together with microglia, are the main producers of APOE in the CNS [84], but its expression and secretion are isoform- and cell-manner in the presence of inflammation. Notably, the isoform APOE-ε4 human astrocytes exhibit a pro-inflammatory profile in human astrocytes, characterized by heightened activity of the NF-κB pathway [85]. For all these reasons has emerged as a significant genetic risk factor for SAD [86]. Regarding the role of pTau metabolism, it is well known that in AD neurons exhibit Tau pathology, but there have been observations of thorn-shaped astrocytes containing perinuclear Tau deposits, particularly in models related to Tau astrogliopathy [87]. A recent study, conducted by Smith and colleagues, analyzed the transcriptome of post-mortem brain tissue from individuals with AD and found a link between the expression of specific genes in glial cells, for example the genes for clustering, myocyte enhancer factor 2C and IQ domain-containing protein K (a

calcium binding protein whose function remains unclear) and the presence of both Aβ plaques or pTau pathology [88].

1.3.2.2 Astrocytes, neuroinflammation and oxidative stress in AD

As already mentioned, in AD astrocytes are important also because of their role on neuroinflammatory processes. They achieve this by triggering intracellular pathways, resulting in oxidative stress and the release of proinflammatory cytokines [60]. Moreover, RA can lead to increased cytotoxicity and oxidative stress, which is linked to the production and aggregation of Aβ plaques in the context of AD. It is also known that Aβ1–42 can increase ROS levels in astrocytes by binding the RAGE and activating NOX complex [89]. In AD, the interactions between astrocytes and microglia play a crucial role in the pathophysiological processes. Experimental models of neuroinflammation have revealed that glial activation occurs at various stages, first microglia were activated and followed by a subsequent activation response in astrocytes. This sequential activation suggests a dynamic interplay between these two types of glial cells in response to AD-related pathology. Microglia, as the brain's primary immune cells, often initiate the immune response by responding to pathological changes early in the disease process, and astrocytes subsequently become activated to participate in the ongoing neuroinflammatory cascade [77]. As mentioned before, oxidative stress and mitochondrial dysfunction are typically involved in AD, potentially resulting in synaptic damage induced by Aβ. In fact, an increase in Aβ levels is directly associated with elevated ROS levels [28]. High concentrations of ROS can induce a neurotoxic state in astrocytes, primarily through the activation of inducible nitric oxide synthase (iNOS), leading to nitrosative stress and detrimental nitration processes in neurons [90]. The activation of astrocytic iNOS in response to Aβ appears to rely on the presence of

IL-1β, TNF, mediated by a signaling pathway dependent on NF-κB-inducing kinase (NIK) [91]. These findings suggest a strong correlation between the balance of neurotoxic and neuroprotective responses by astrocytes and the extent of ROS production. A persistent feedback loop exists between neuroinflammation and oxidative stress, wherein one of these abnormal processes triggers the other, creating a self-sustaining cycle.

1.3.2.3 Astrocytes and gliotransmitters in AD

Astrocytes play a significant role in gliotransmission and the modulation of excitotoxicity in AD. Gliotransmission refers to the communication between astrocytes and neurons through the release of signaling molecules, such as neurotransmitters and neuromodulators. In the context of AD, astrocytes can influence the balance of excitatory and inhibitory neurotransmission, impacting neuronal function [92]. One aspect of astrocyte involvement in AD relates to the regulation of glutamate, a major excitatory neurotransmitter. This regulation is largely carried out by astroglial transporters, with two of the most prominent ones being Glutamate Transporter-1 (GLT-1) and Glutamate Aspartate Transporter (GLAST). GLT-1 and GLAST are responsible for efficiently clearing excess glutamate from the extracellular space in the brain. This function is crucial because excessive glutamate can lead to a phenomenon known as excitotoxicity, where neurons become overstimulated and damaged [93]. In AD, disruptions in glutamate clearance mechanisms can contribute to this excitotoxicity and subsequent neuronal harm [94]. Research has shown that in AD, there can be alterations in the expression and function of GLT-1 and GLAST in astrocytes. These changes may lead to impaired glutamate uptake and clearance, thereby exacerbating the excitotoxic effects associated with the disease [93]. Additionally, the accumulation of Aβ

plaques can further disrupt glutamate homeostasis and impact the function of these transporters [95].

1.3.2.4 The crosstalk between astrocytes and neurons in AD

The intricate cross talk between astrocytes and neurons takes on a particularly crucial role in the context of AD [96] (Fig. 5). Astrocytes, often dubbed the "caretakers" of the brain, play a multifaceted role in maintaining neuronal health, including the regulation of synaptic activity and neurotransmitter balance as previously mentioned, considering that brain glucose metabolism is fundamental in AD. The brain's high energy demands, particularly in regions associated with memory and cognition, make it especially sensitive to disruptions in glucose metabolism. Astrocytes, as the primary regulators of energy supply, help bridge the gap between glucose availability and neuronal needs in AD. Astrocytes step in to provide an alternative energy source through lactate production, which neurons can efficiently use, thereby sustaining their ATP levels and overall vitality [64]. This compensatory mechanism underscores the adaptive nature of astrocytes in the face of AD-related metabolic disturbances. Both astrocytes and neurons have the capability to effectively metabolize glucose and lactate. However, it's worth noting that astrocytes exhibit a more pronounced glycolytic profile, while neurons tend to favor oxidative metabolism through mitochondrial OxPhos [97]. This distinction in metabolic preferences laid the foundation for the development of the astrocyte-neuron lactate shuttle (ANLS) hypothesis. This hypothesis gained support from research studies indicating incomplete glucose oxidation and/or heightened lactate production as a response to increased neuronal activity. In addition, astrocytes play a vital role in rapidly removing neurotransmitters like glutamate from the synaptic cleft. They achieve this through high-affinity sodium-dependent glutamate

transporters. The recycling of glutamate by astrocytes is essential because neurons lack the necessary enzymes for de novo synthesis of glutamate [98,99]. Astrocytes can convert this glutamate into glutamine, a non-neuroactive compound, which is then safely transported back to neurons. There, it can be converted back into glutamate when needed. In a similar approach, at GABAergic synapses, astrocytes take up released GABA, and it is processed in mitochondria through the TCA cycle. Glutamine produced by astrocytes can also be utilized by inhibitory GABAergic neurons for GABA synthesis. Both neurons and astrocytes can utilize glutamate and glutamine for other metabolic purposes, such as energy production or nitrogen balance regulation. The net removal of these compounds is balanced through a process called anaplerosis, facilitated by the astrocyte-specific enzyme pyruvate carboxylase [97]. Impaired astrocyte metabolism can trigger an inflammatory response within the brain. This inflammation can have detrimental effects on neuronal health and viability and is triggered by various factors, including impaired astrocyte metabolism, which can result in the release of proinflammatory molecules within the brain. These molecules can exacerbate the damage to neurons and contribute to the worsening of cognitive decline. In neurodegenerative diseases like AD, chronic neuroinflammation is a hallmark feature, and it can lead to a vicious cycle of neuronal damage and cognitive impairment. Understanding and addressing these disruptions in astrocyte-neuron communication are crucial aspects of research aimed at finding effective treatments and interventions for AD.

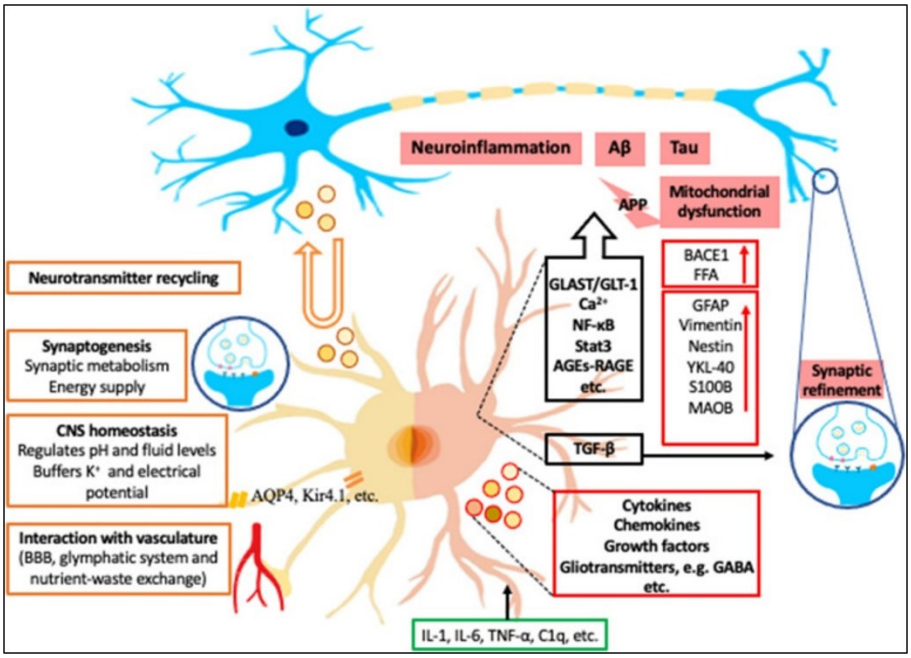


Figure 5: The crosstalk between astrocytes and neurons in AD [66]

1.4 The GA in vitro model

As already mentioned in the previous paragraph relating to AGEs, these compounds are actively involved in the pathogenesis of AD. A study conducted by Koriyama and colleagues demonstrated that SH-SY5Y neuroblastoma cells treated with GA induce an increase of AGEs, which subsequently disrupt the levels of AD biomarkers and decrease cell viability [45]. This research effort has enabled my research group to create an in vitro Alzheimer's model using GA, which can replicate the prodromal characteristics typically seen at the beginning of the disease [100,101]. Initially, this model was employed in SH-SY5Y cells differentiated with retinoic acid and then in primary rat cortical neurons. This approach enabled us to evaluate how GA affected cells from multiple perspectives. Our findings revealed several significant effects. Firstly, we found out that GA exposure led to a decrease in cell viability, in a time and concentration dependent manner. GA exposure also increases the two main AD markers, Aβ and pTau. The increase in Aβ1-42 accumulation is probably

due to the fact that APP is compromised, potentially leading to the generation of toxic amyloidogenic fragments during metabolic dysfunction. In particular, Aβ1-42 also accumulated within mitochondria, potentially due to impaired interactions between neurotoxic Aβ oligomers and alterations in mitochondrial function [101]; APP and Aβ are capable to enter mitochondria, where they can engage with various mitochondrial components, and in turn, can damage ATP synthesis while simultaneously promoting the accumulation of ROS [102]. We also analyzed another aspect, energy metabolism, which was severely affected by GA, by monitoring two critical parameters: mitochondrial respiration, evaluated through cellular oxygen consumption rates (OCR), and glycolysis, determined by measuring extracellular acidification rates (ECAR). GA had a detrimental impact on cellular bioenergetics, leading to a significant reduction in both mitochondrial respiration and glycolysis, consistent with the inhibitory effects of GA. These observations provided deeper insights into overall mitochondrial function, indicating impairments in mitochondrial membrane potential (Δψm) and changes in proton leakage, both heavily influenced by GA treatment [100,101]. In our research, we noted an increase in intracellular and mitochondrial ROS in cells treated with GA [100,101,103]. Furthermore, our observations revealed a significant reduction in Superoxide Dismutase activity induced by GA, shifting the cellular redox balance toward pro-oxidative conditions [101]. We observed that the disruption in oxidative balance and the alterations in mitochondrial functions resulting from GA treatment are linked to the disruptions in both cytosolic and mitochondrial Ca^{2+} homeostasis. The elevated concentrations of mitochondrial Ca^{2+} could be linked to the decline in ΔΨm and, concurrently, may intensify the imbalance between pro-oxidant and antioxidant defenses, potentially leading to the generation of mitochondrial ROS [100,101,103]. As the final step, we investigated the death pathways implicated in the GA model, considering the link

between oxidative stress (as well as the presence of toxic AGEs) and the (AMP)-activated protein kinase (AMPK)-mammalian target of rapamycin (mTOR) pathway [104]. The AMPK-mTOR pathway plays a critical role in the regulation of various cellular processes, including energy metabolism, cell growth, and autophagy. AMPK exhibits a multifaceted role. Its activity is observed to decrease in AD [105] while it has also been demonstrated to play a neuroprotective role against cytotoxicity induced by AGEs [106]. mTOR, which is negatively regulated by AMPK, plays a pivotal role in regulating cell growth, protein synthesis, and survival. In neurons, inhibiting mTOR during conditions of stress or damage can be neuroprotective. Excessive mTOR activity can promote neurodegeneration by stimulating protein synthesis and inhibiting autophagy. Dysregulation of mTOR signaling can lead to various diseases and disorders [107]. In mTOR pathway one of the key downstream components p70 ribosomal S6 kinase (p70S6K) is a pivotal player in AD pathology. Its involvement in AD is multifaceted and encompasses the phosphorylation of Tau protein and the regulation of Aβ production [108]. We evaluated that GA challenge induce a reduction of AMPK expression and an upregulation of mTOR. These findings provide support for the existence of multiple interconnected molecular mechanisms that collectively form a vicious cycle, driving the neurodegenerative processes associated with AD.

2 Aim of this study

Astrocytes play a crucial role in AD, significantly influencing neighboring cells. In this neurodegenerative context, astrocytes not only actively support neuronal homeostasis but can also contribute to the progression of the pathology. In AD, astrocytes may exhibit dysfunctions compromising their capacity to maintain ion balance and clear toxins in the brain. These alterations directly impact nearby cells, including neurons, escalating inflammation and contributing to the formation of neuronal plaques, thereby advancing the disease. Exploring the intricate connections that exist between astrocytes and neurons in the context of neurodegenerative conditions represents a highly promising avenue for developing potential therapeutic interventions. Thus, the aim of this study was to investigate the metabolic crosstalk between astrocytes and neurons in the development of AD. In particular, this work explored how the metabolic dysfunction arising from astrocytes may affect the neighboring neurons. To achieve this, an in vitro model capable of replicating the prodromal stages of the disease, (including a condition of hypometabolism and mitochondrial dysfunction) was used in a cocolture of neurons and astrocytes. The focus was on the potential benefits of metabolic interventions, by using alternative substrates to glucose, with a specific focus on glutamate,aimed to restore the energetic balance in astrocytes and their possibility to positively influence neuronal vitality.

3 Materials and methods

3.1 Cell culture and treatments

Cell Culture: Primary rat cortical astrocytes (from this point onward, cortical astrocytes) and neurons (cortical neurons) were isolated from Wistar (Cat. 003WISTAR, Charles River, Lecco, Italy) rat pups (P2–P4). Ethical guidelines were strictly followed, ensuring compliance with the Ethics Committee for Animal Experiments, and adhering to the Italian Ministry of Health's regulations. Cortices were isolated from rat brains and placed in ice-cold phosphate-buffered saline (PBS). The PBS was replaced with trypsin-EDTA (0.25% for cortical astrocytes and 0,05% for cortical neurons) and subsequently the cortices were subjected to mechanical dissection by scissor cut, followed by enzymatic digestion obtained by incubating them for 15 minutes in a humidified incubator at 37°C with atmosphere at 5% CO_2. After trypsinization, the cells were centrifuged at 2000 rpm for 5 minutes. To neutralize the trypsin, the cortices were washed with media Dulbecco's Modified Eagle Medium (DMEM, Corning, New York, USA) and centrifuged using the same program. Cortical astrocytes were washed with DMEM and triturated with a fire-polished glass pipette 20 times and then seeded in T-25 flask. After removing the supernatant, cortical neurons were triturated using a 5 ml syringe with an 18 G needle 3 times and then centrifuged. This step was repeated twice, and then cortical neurons were plated on poly-D-lysine-coated glass coverslips. Cortical astrocytes were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS, Corning), 100 U/ml penicillin, and 100 µg/ml streptomycin (Corning). Cortical neurons were maintained in DMEM supplemented with B27, 100 U/ml penicillin, 100 µg/ml streptomycin (Corning) and 2 mM glutamine (Corning). Cells were cultured at 37 °C in an atmosphere with 5% CO_2. Experiments were performed between days 10 and 14 in vitro (DIV).

Treatments: To induce AD-like alterations, cells were exposed to GA (1 mM), which creates a condition of hypometabolism accompanied by mitochondrial dysfunction and redox imbalance, thus reproducing an environment often observed in the early stages of AD. To investigate the role of glutamate 1mM (Glut) as an alternative metabolic substrate, glutamate was added after 24 and 40 h of GA exposure in astrocytes before the coculture setting.

Cocultures of astrocytes and neurons: The cocultures (CC) were set by plating cortical astrocytes onto poly-D-lysine coated 6 wells plate. When a monolayer of cortical astrocytes was obtained, cells were treated with GA. After 48 hours cortical astrocytes medium was removed, cortical neurons plated on a coverslip were added and maintained with cortical astrocytes for 24 hours. At the end of the experimental protocol, cells were harvested for further analysis.

3.2 Cell Viability

Cell viability was evaluated through the MTT assay, LDH assay and by the method of double staining with fluorescein diacetate/propidium iodide (FDA/PI). The MTT assay evaluated the mitochondria's capability to metabolize the yellow tetrazolium salt, 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), resulting in the creation of insoluble purple formazan crystals. After incubation with MTT solution (0.5 mg/mL in PBS) for 1 hour in a dark, controlled environment at 37 °C and 5% CO_2, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St.Louis, MO, USA). The amount of generated formazan was directly proportional to the cells' mitochondrial activity, and the absorbance value was quantified at a wavelength of 540 nm using a Victor Multilabel Counter plate reader from Perkin Elmer (Waltham, MA, USA). A reduction in mitochondrial activity led to a corresponding decrease in the observed absorbance value. The outcomes

were presented as percentages relative to the control value. LDH assay was assessed to evaluate the measurement of LDH activity released from the cytosol of damaged cells in the experimental media. After following the experimental protocol, 50 microliters of the culture medium were obtained and dispensed into a 96-well plate. The medium was then subjected to incubation with the Cytotoxicity Detection Kit reaction solution (Roche, Basilea, Switzerland) for 30 minutes. Subsequently, the optical density was measured at a wavelength of 490 nanometers using a Victor Multilabel Counter plate reader. To perform FDA/PI staining, cells were first cultured on glass coverslips and exposed to GA. Subsequently, the cells were treated with 36 μM of FDA (Sigma, Milan, Italy) and 7 μM of PI (Calbiochem, San Diego, CA, USA) for 10 minutes at room temperature (RT) in PBS. Following staining, the cells were observed using an inverted 510 LSM microscope (Carl Zeiss, Milan, Italy) and then subjected to analysis. The staining process involves FDA, which, upon penetrating the cell membrane, undergoes hydrolysis by intracellular esterases, resulting in the emission of a green-yellow fluorescence. Cell damage limits the effectiveness of FDA staining, allowing PI to permeate the cells instead. PI interacts with nuclear DNA, leading to the emission of a vivid red fluorescence.

3.3 Evaluation of ATP Levels

Intracellular ATP levels were analyzed by using a commercially luciferase-luciferin kit (ATPlite, Perkin Elmer). In brief, cortical astrocytes were plated on 96-well ViewPlate (Perkin Elmer) and then exposed to the indicated treatments in DMEM medium. ATP levels were evaluated with a luminescence counter (Victor Multilabel Counter, Perkin Elmer), normalized to their corresponding protein content, and presented as percentages relative to the control value.

3.4 Measurement of Mitochondrial ROS Production

Mitochondrial ROS production was observed using the MitoTracker CM-H2XRos (Invitrogen Life Technologies, Carlsbad, CA, USA) dye. In brief, cells were seeded on coverslips (poly-D-lysine-coated for cortical neurons) and subjected to distinct treatments. After the experimental protocol, the cells were exposed to a 300 nM dye concentration for 30 minutes at 37°C, and washed 3 times with PBS. Confocal images were captured using a 510 LSM microscope (Carl Zeiss) with a META detection system. The excitation of CM-H2XRos occurred at 560 ± 10 nm, and emission was measured at 620 ± 20 nm. Image acquisition transpired every 5 seconds, allowing for the tracking of basal ROS levels for roughly 200 seconds. Post-image acquisition, fluorescence intensity analysis was performed offline. The fluorescence values were expressed as percentages relative to the control value.

3.5 Immunocytochemistry

Immunofluorescence staining. Cells, after the protocol experiment, were exposed to MitoTracker Red CMXRos (Invitrogen) at a concentration of 300 nM for 30 minutes at a temperature of 37°C. Next, they were fixed using PBS and 3.7% formaldehyde for another 30 minutes at room temperature. After this fixation, the cells were permeabilized with PBS containing TritonX-100 for a duration of 5 minutes at RT. The cells were subjected to a 1.5-hour incubation at RT with primary antibodies, either Aβ1-42 or pTau AT100. To identify the immunoreactions, a conjugated secondary antibody (Alexa Anti-Mouse 488 Thermo Scientific) with a 1:200 dilution, was employed.

Primary antibodies. Aβ1-42 and pTau proteins were used with a dilution of 1:100 in PBS with 1% BSA (Aβ1-42: mouse monoclonal IgG1 antibody, clone 12F4, Cat. 805501,

Biolegend, San Diego, CA, USA); (pTau: human PHF-Tau monoclonal IgG, clone AT100, Thermo Scientific).

3.6 Enzyme-linked Immunosorbent Assay (ELISA)

The evaluation of TNFα release was conducted using the Rat TNF alpha ELISA Kit (Invitrogen). After subjecting astrocytes to GA treatments, the supernatant was collected. Subsequently, the levels TNF-α were assessed through ELISA kits, following the manufacturer's guidelines. TNFα levels were evaluated with a Victor Multilabel Counter (Perkin Elmer) by reading the absorbance at 450 nm and quantified using their respective standard curves.

3.7 Western Blot

Total lysates of cortical astrocytes were prepared in B-buffer 1X, (composed in mM, with 150 NaCl, 10 Tris-HCl, 1 EDTA, and 1% SDS). The protein content was determined using the Bradford method (Bio-Rad, Milan, Italy). Samples containing equal protein amounts (40 µg) were prepared in 6x Laemmli sample buffer with 2-mercaptoethanol and boiled for 10 minutes. These proteins were then separated via electrophoresis on an 8% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). Following the blocking phase (1 hour at RT with 5% non-fat dry milk in PBS buffer), the membranes were incubated overnight at 4°C with the appropriate primary antibody. To detect the immunoreactions, the membranes were subsequently incubated with the suitable secondary antibody, which was conjugated to horseradish peroxidase (Goat anti-rabbit IgG-HRP, Santa Cruz, CA, USA, Goat anti-Mouse IgG (H + L) HRP Cat. 62-6520, Thermo Scientific), for 1 hour at RT. Blots were developed using an enhanced chemiluminescence detection kit

(Clarity Max Western ECL Substrate, BioRad), and images were captured using a Uvitec Cambridge Chemiluminescence Imaging System (Cambridge, UK).

Primary antibodies: anti-p-NF-κB (Santa Cruz), and anti-β actin (Santa Cruz), anti-GLT-1 and anti-GLAST (Immunological Sciences, Rome, Italy). Band densities were analyzed with Uvitech Nine Alliance analysis software.

3.8 Drugs and Chemicals

Glyceraldehyde was obtained from Santa Cruz. All the remaining chemicals were obtained from Sigma.

3.9 Statistical Analysis

Data were expressed as mean ± standard error of the mean (S.E.M). Statistical analyses of the findings were carried out using GraphPad Prism® 5 software (San Diego, CA, USA). To identify variations among the experimental groups, a one-way ANOVA analysis was performed, followed by Dunnett's post hoc test. To compare the mean of two groups was used the unpaired t-test. Statistical significance was considered achieved when $p < 0.05$.

4 Results

4.1 Effect of GA on the viability of cortical astrocytes

To induce a condition of hypometabolism, cortical astrocytes were exposed to GA (1 mM), as previously described [100]. We first determined the effect of GA at different timing by treating cells for 24, 32 and 48 h. Cortical astrocytes viability was significantly reduced after 48h of GA exposure by evaluating LDH release in culture medium (Fig. 6A). Mitochondrial activity was also evaluated, which was significantly reduced at 24, 32 and 48 h, as assessed by the MTT assay (Fig. 6B). The 48 h treatment with GA was then used for the subsequent experiments.

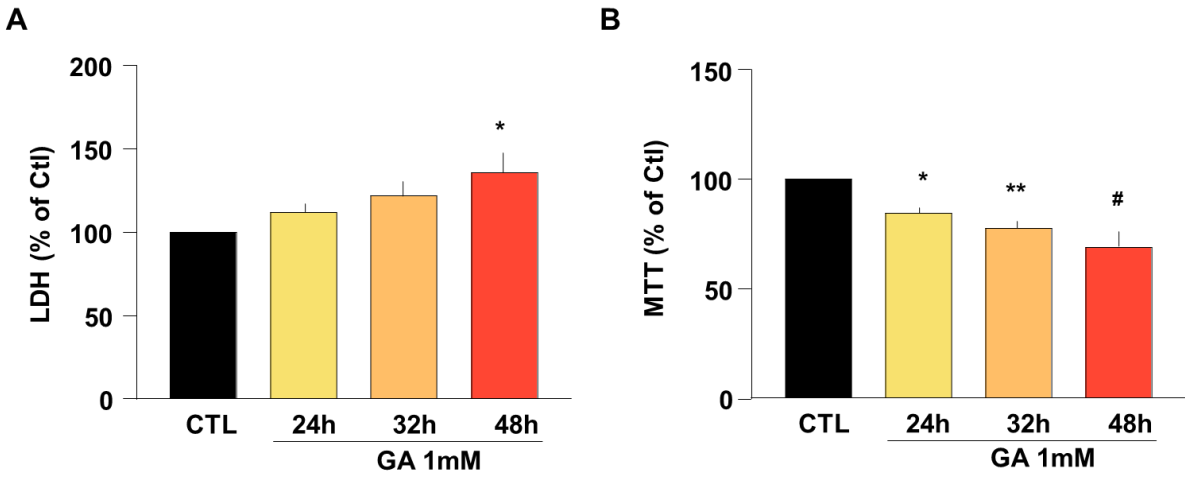


Figure 6: Effect of GA on the viability of primary rat cortical astrocytes. Effect of GA (1 mM) on astrocytes viability assessed with LDH assay (A) and MTT assay (B). The cells were exposed to GA for different timing, 24, 32 and 48 h. In each experiment extracellular LDH release and MTT reduction were expressed as a percentage of the control. Each column represents the mean ± S.E.M. of at least 3 experiments performed in triplicate. Significant differences were evaluated by one-way ANOVA followed by Dunnett's post hoc test. A $F(3, 16) = 4.033$. * Significant vs CTL ($p < 0.05$). B $F(3, 28) = 15,69$. * Significant vs CTL and

GA 48 h ($p < 0.01$). ** Significant vs CTL ($p < 0.001$). # Significant vs CTL and GA 24 h ($p < 0.0001$ vs CTL, $p < 0.01$ vs GA 24 h). CTL control, GA glyceraldehyde.

4.2 Effect of 48 h exposure of GA on mitochondrial ROS formation and ATP production in cortical astrocytes

Elevated levels of mitochondrial ROS in primary astrocytes can cause oxidative stress, leading to detrimental consequences that may involve the neighboring cells. For instance, the ROS-induced release of pro-inflammatory cytokines may trigger neuroinflammation, causing mitochondrial dysfunction, which in turn may result in a decrease in energy production, thus feeding a vicious cycle of ROS generation and cellular damage, and contributing to neurological disorders. Reactive astrocytes may undergo a process called gliosis in response to high ROS levels and neuroinflammation, which involves changes in astrocyte morphology and gene expression, which can impact their supportive functions within the CNS. Based on this premise, we analyzed the possible effect of the GA exposure on the production of ROS and ATP, in order to better elucidate the mechanisms of primary astrocytes death and to explore the role of astrocytes in adjacent cells, including neurons, in order to examine their responses to alterations in the environment. Interestingly, the exposure to GA significantly increased the levels of mitochondrial ROS (Fig. 7A, B) and ATP levels (Fig. 7C) in cortical astrocytes.

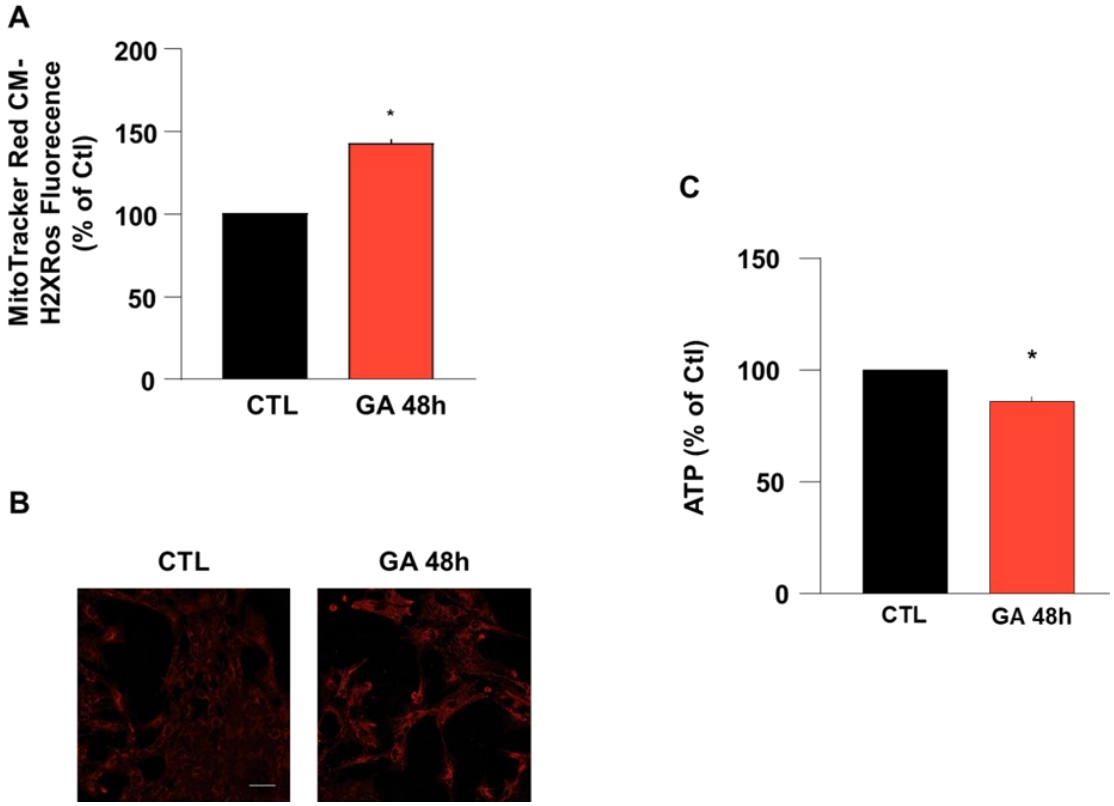


Figure 7: Effect of GA treatment on mitochondrial ROS and ATP production in cortical astrocytes. Quantification of mitochondrial ROS and ATP levels in cortical astrocytes challenged with GA (1 mM) for 48 h and reported as percentages relative to the respective control group. Each column represents the mean ± S.E.M. of at least 5 experiments performed in triplicate. Images displaying mitochondrial ROS fluorescence (B) levels, representative of 5 experiments (50–100 cells for each experimental group were analyzed). Scale bar 50 μm. Differences among means were assessed by Student's t-test. A * $p < 0.0001$ vs CTL, C * $p < 0.001$.

4.3 The exposure to GA induced an inflammatory response in cortical astrocytes

Under specific conditions, astrocytes exhibit a remarkable capability to undergo activation and, in doing so, can initiate an inflammatory response which arises in response to various external stimuli or internal cues. When triggered, activated astrocytes release pro-inflammatory molecules and engage in intricate cellular signaling pathways that orchestrate an immune response within the brain. 48 h of GA treatment activated an inflammatory response in cortical astrocytes. This response was characterized by the significant upregulation of NF-κB (Fig. 8A, B), a transcription factor responsible for the expression of pro-inflammatory cytokines and chemokines [109]. Furthermore, this activation led to a subsequent and significant elevation in the levels of the pro-inflammatory cytokine TNF-α in the culture medium (Fig. 8C).

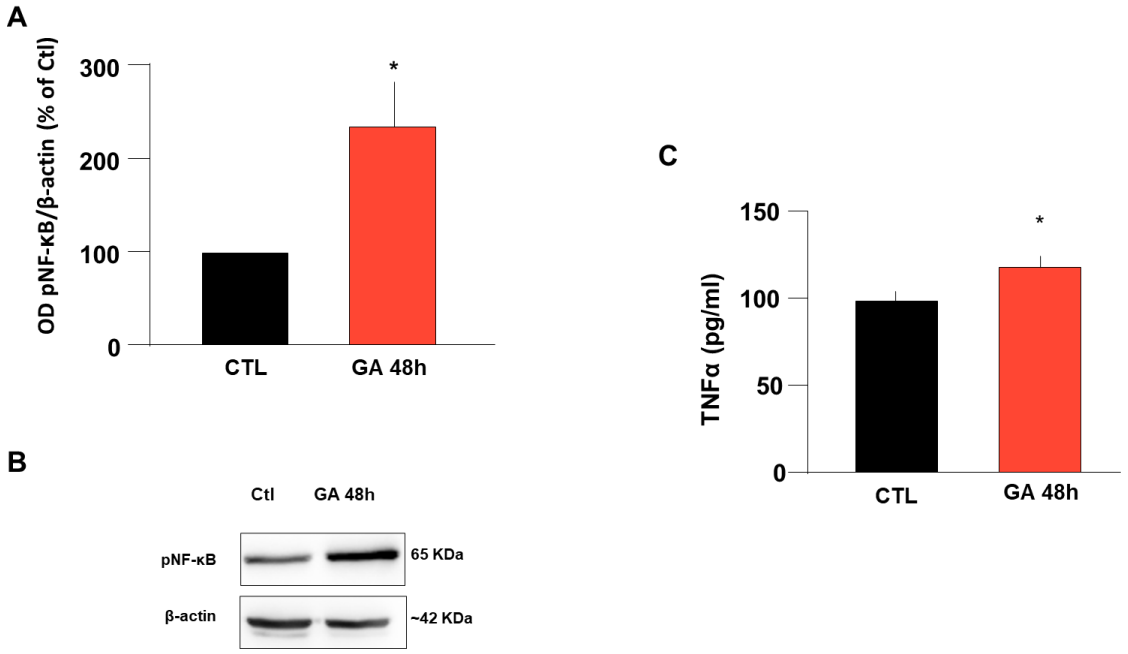


Figure 8: Effect of GA on inflammatory response in cortical astrocytes. Quantification of NF-kB phosphorylation (pNF-κB) expression (A, B) and evaluation of TNFα levels (C) in cortical neurons challenged with GA (1 mM) for 48 h. For the expression of pNF-κB,

β-actin was used as a loading control. Normalized optical density values were reported as control percentages. Representative western blot images are shown above. Differences among means were assessed by Student's t-test. A * $p < 0.05$ vs CTL. B * $p < 0.05$.

4.4 Glutamate-induced recovery of ATP synthesis in GA treated cortical astrocytes

After confirming the adverse effects of GA treatment on cortical astrocytes, the study proceeded in order to evaluate whether glutamate, used as an alternative substrate, could be able to improve ATP production in cells. Glutamate was added after 24 and 40 hours of GA exposure, and in both time frames and in control conditions it significantly increased intracellular ATP synthesis. In particular, when added during GA challenge, the intracellular levels of ATP were restored to control levels (Fig. 9A, B).

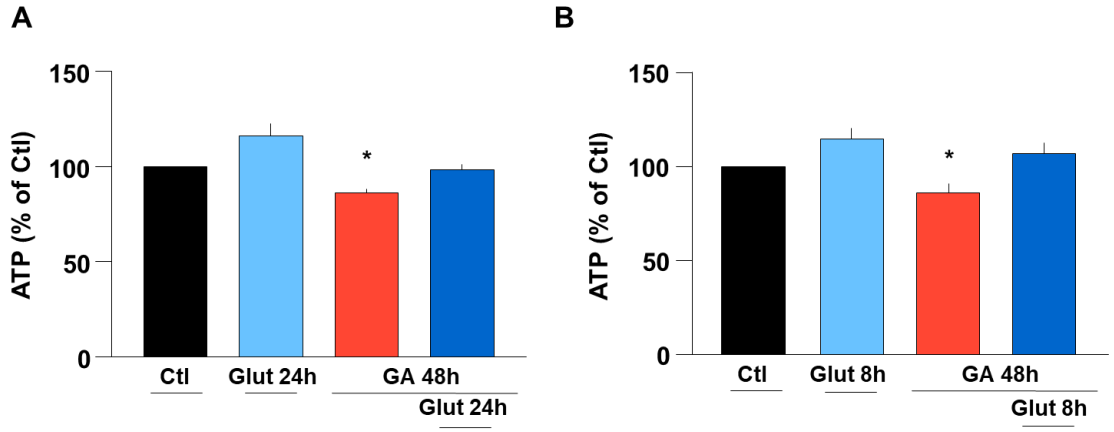


Figure 9: Effect of glutamate on intracellular ATP levels in cortical astrocytes exposed to GA. Glutamate was added after 24 h (A) and 40 h (B) of GA (1 mM) treatment for 48 h. Cell viability results, determined as measurements of intracellular ATP levels are graphed the column charts and reported as percentages relative to the respective control groups. Each column represents the mean ± S.E.M. of at least 3 experiments performed in triplicate. Significant differences were evaluated by one-way ANOVA followed by Dunnett's post hoc

test. A F (3, 32) = 8,351. * Significant vs all groups ($p < 0.05$ vs CTL and GA48h+24hGlut, $p < 0.0001$ vs Glut24h). B F (3, 20) = 16,29. * Significant vs all groups ($p < 0.01$ vs CTL and GA48h+Glut8h, $p < 0.0001$ vs Glut8h).

4.5 GA significantly increased the levels of the glutamate transporters GLT-1 and GLAST in cortical astrocytes

Astrocytes play a crucial role in regulating the levels of neurotransmitters, particularly glutamate, within the central nervous system. Two important transporters responsible for controlling extracellular glutamate levels are GLAST and GLT-1. These transporters are primarily expressed in astrocytes and are responsible for clearing the excess of glutamate from the synaptic cleft, preventing excitotoxicity, and maintaining proper neurotransmission [93]. 48 h of GA exposure induced a metabolic alteration in cortical astrocytes, which is paralleled by an increase in the expression of GLT-1 (Fig. 10A, B) and GLAST (Fig. 10C, D). The alteration of which may have significant implications for energy metabolism.

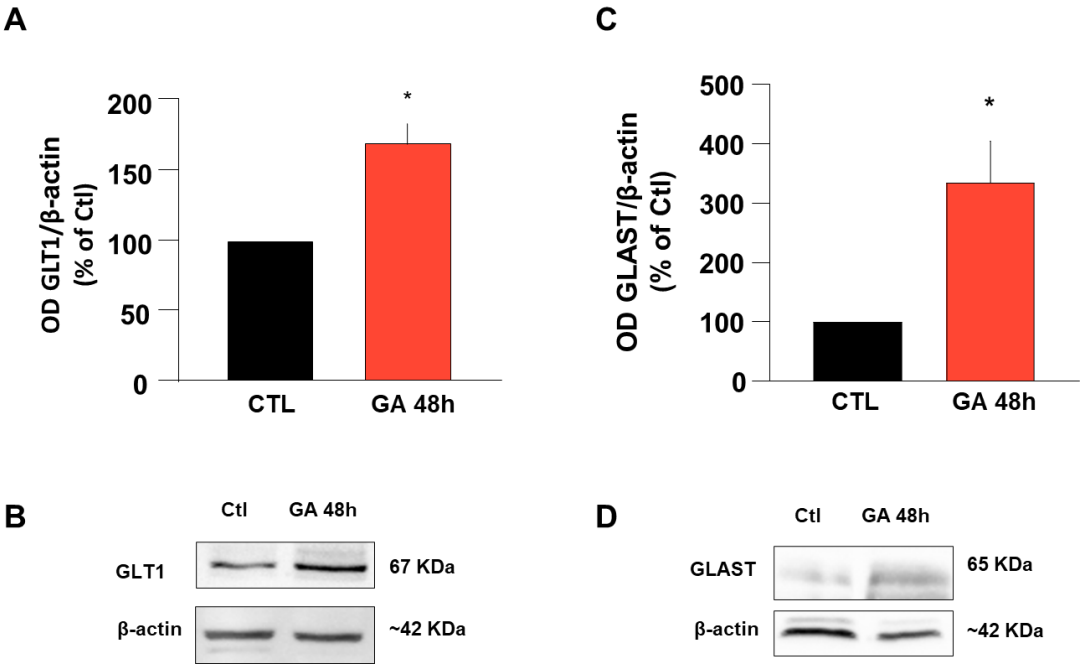


Figure 10: Effect of GA on the glutamate transporters GLT-1 and GLAST in cortical astrocytes. Quantification of GLT1 (A, B) and GLAST (C, D) expression in cortical neurons challenged with GA (1 mM) for 48 h. β-actin was used as a loading control. Normalized optical density values were reported as control percentages. Representative western blot images are shown above. Differences among means were assessed by Student's t-test. A * $p < 0.001$ vs CTL. B * $p < 0.05$.

4.6 Glutamate-induced recovery on ROS production in GA treated cortical astrocytes

Having asserted that glutamate induces an increase in ATP production in cortical astrocytes treated with GA, the role of glutamate in ROS production induced by GA was evaluated as previously demonstrated. As already mentioned, in pathological conditions, RA react to environmental changes by generating free radicals. Glutamate, added after 24 and 40 hours of the 48 hours of GA treatment, had a protective effect by significantly reducing the ROS levels induced by GA (Fig. 11A, B).

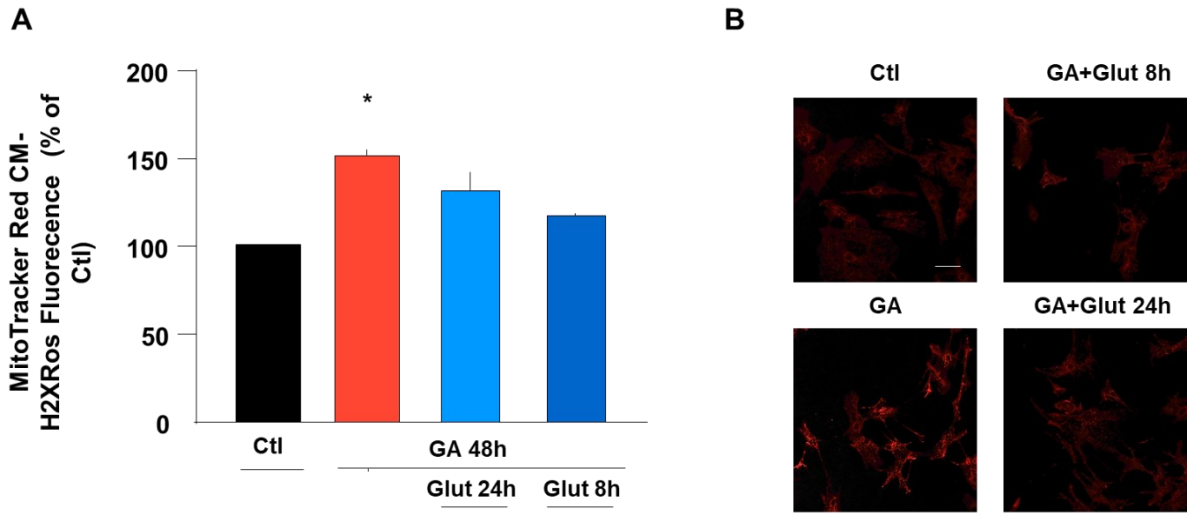


Figure 11: Effect of glutamate on ROS production in cortical astrocytes exposed to GA. Quantification of mitochondrial ROS (A, B) in cortical astrocytes challenged with GA (1 mM) for 48 h and glutamate, added after 24 h and 40 h of GA treatment**.** Values are reported as percentages relative to the respective control group. Each column represents the mean ± S.E.M. of 3 experiments performed in triplicate. Images displaying mitochondrial ROS fluorescence (B) levels, representative of 5 experiments (50–100 cells for each experimental group were analyzed). Significant differences were evaluated by one-way ANOVA followed by Dunnett's post hoc test. A $F(3, 8) = 46{,}60$. *Significative vs all groups ($p < 0.0001$ vs CTL, $p < 0.01$ vs GA48h+Glut24h, $p < 0.001$ vs GA48h+Glut8h).

4.7 Glutamate-induced recovery on inflammatory state in GA treated cortical astrocytes

Considering that, in cortical astrocytes, GA stimulates oxidative stress and an inflammatory response, and that glutamate reduces the increase in mitochondrial ROS levels, the effect of glutamate on the inflammatory state was evaluated. Glutamate, added after 24 and 40 hours of the 48 hours of GA treatment, had a protective effect by mitigating the inflammatory response induced by GA. In particular, glutamate significantly reduced the expression of pNF-κB (Fig. 12A, B) and the levels of the pro-inflammatory cytokine TNF-α in the culture medium (Fig. 12C).

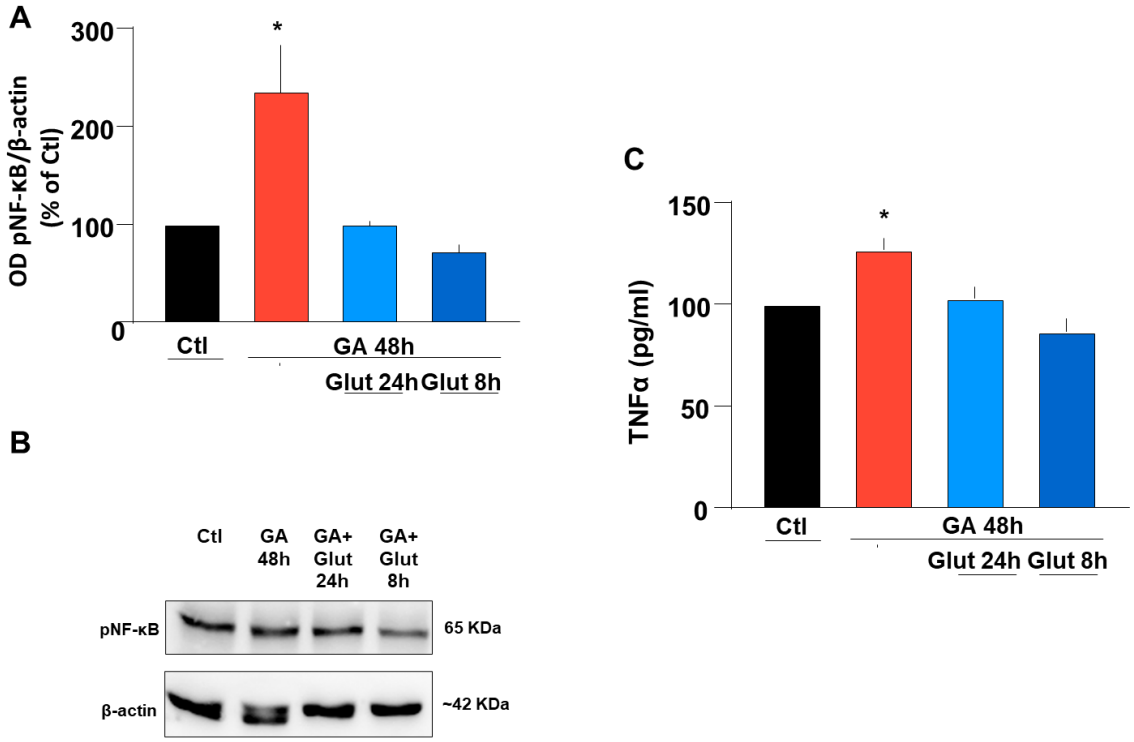


Figure 12: Effect of glutamate on ROS production and inflammatory response in cortical astrocytes exposed to GA. Quantification of pNF-κB expression (A, B) and evaluation of TNFα levels (C) in cortical astrocytes challenged with GA (1 mM) for 48 h and glutamate, added after 24 h and 40 h of GA treatment**.** Values are reported as percentages relative to the respective control group. Each column represents the mean ± S.E.M. of 3 experiments performed in triplicate. For the expression of pNF-κB, β-actin was used as a a loading control. Normalized optical density values were reported as control percentages. Representative western blot images are shown above. Significant differences were evaluated by one-way ANOVA followed by Dunnett's post hoc test. A $F(3, 18) = 5{,}954$. * Significative vs all groups ($p < 0.01$ vs CTL and GA48h+Glut8h, $p < 0.05$ vs GA48h+Glut24h). C $F(3, 14) = 13{,}89$. * Significative vs all groups ($p < 0.001$ vs CTL and GA48h+Glut8h, $p < 0.05$ vs GA48h+Glut24h).

4.8 Cortical neurons viability was significantly reduced after 24 h of coculture with cortical astrocytes previously challenged with GA

By using the system of CC between primary astrocytes and neurons the viability of neurons was evaluated. We found that the altered metabolism of primary astrocytes treated with GA induced a cascade of events that negatively influenced neuronal viability, which in fact was significantly reduced after being cocultured with primary astrocytes previously treated with GA for 48 hours, assessed by the method of double staining with FDA/PI (Fig. 13A) and by the MTT assay (Fig. 13B). The findings provide strong support for the hypothesis that impaired astrocytes indeed have a detrimental influence on adjacent cells, like neurons.

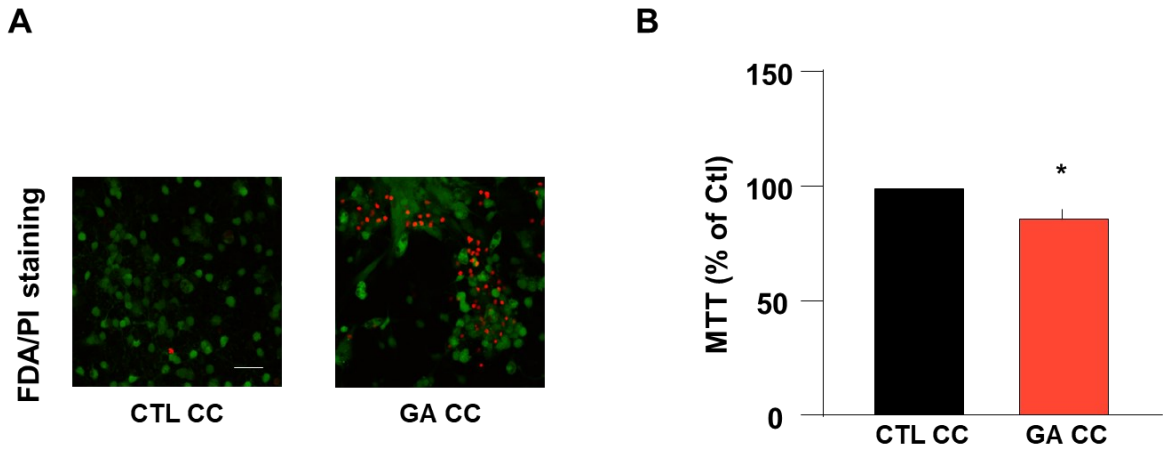


Figure 13: Effect of GA on cortical neurons viability cocultured with cortical astrocytes previously challenged with GA for 48 h. Effect of GA (1 mM) on neuronal cell viability assessed with FDA/PI staining (A) and MTT assay (B). Images are representative of three independent experiments. Scale bar 50 µm. (B) Cell viability was assessed by means of MTT assay and reported as percentages relative to the control group. Each column represents the mean ± S.E.M. of 7 experiments performed in triplicate. Differences among means were assessed by Student's t-test. B * $p < 0.05$ vs CTL.

4.9 pTau and Aβ levels were significantly increased in cortical neurons after 24 h of coculture with cortical astrocytes previously challenged with GA.

Accumulation of Aβ and the presence of pTau are distinctive hallmarks observed in the brains of individuals affected by AD. Our research group has revealed that the exposure of cells (both SH-SY5Y and primary rat cortical neurons) to GA results in a lasting modification of these two key biomarkers, coinciding with the overall cellular damage. In cortical neurons, the levels of pTau (Fig. 14A, B) and Aβ (Fig. 14C, D) were significantly increased after being cocultured for 24 h with cortical astrocytes challenged with GA for 48 h. The data obtained on elevated markers of AD are consistent with the decrease in neuronal viability observed in the previously obtained results.

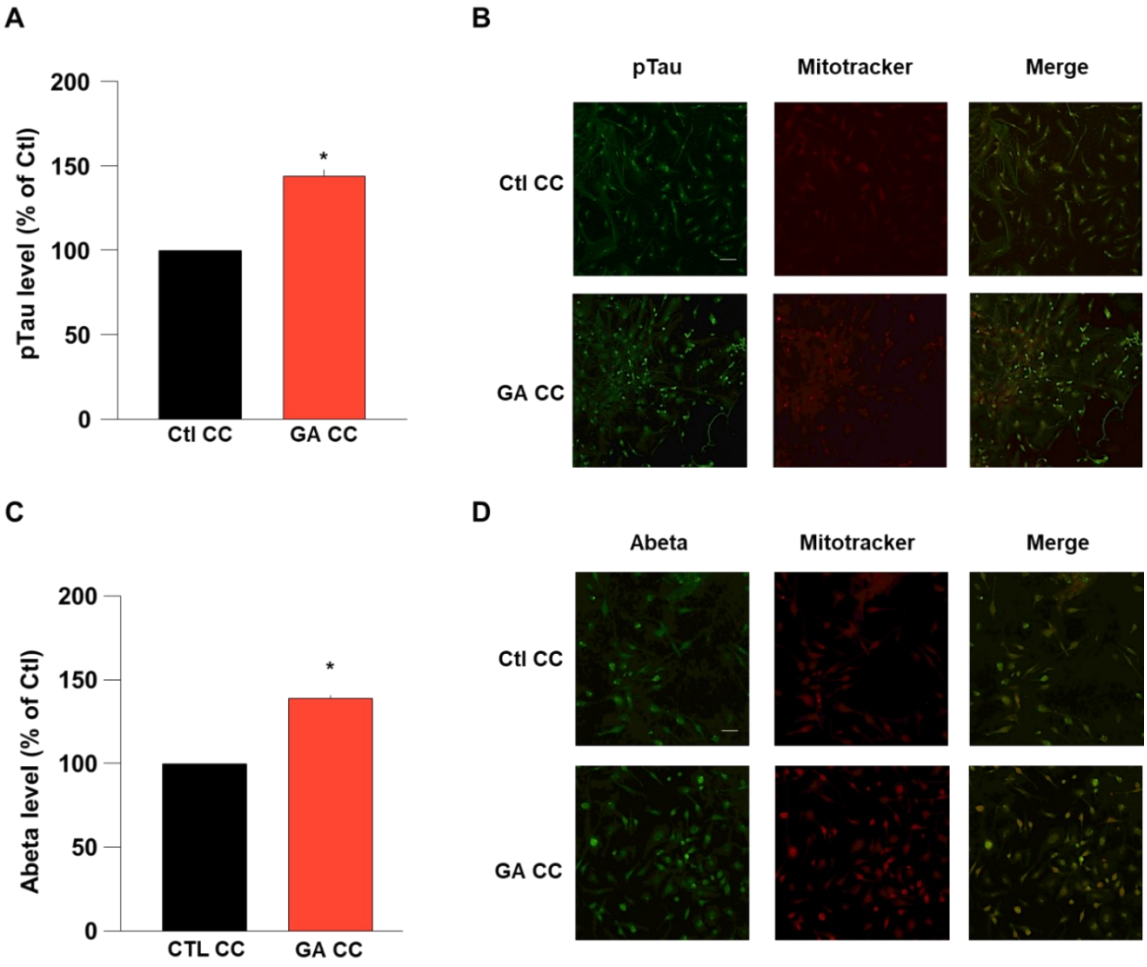


Figure 14: pTau and Aβ expression in cortical astrocytes challenged with GA for 48 h. Quantification (A, C) and representative images (B, D) of pTau and Aβ expression in cortical neurons after 24 h of coculture with cortical astrocytes previously treated for 48 h with GA (1 mM) treatment. pTau and Aβ were detected by immunofluorescence staining. In each experiment the intensity of fluorescence of pTau and Aβ were reported as control percentages. Scale bar 50 μm. Each column represents the mean ± S.E.M. of at least 3 experiments performed in triplicate. Differences among means were assessed by Student's t-test. A * $p < 0.0001$ vs CTL. C * $p < 0.0001$ vs CTL.

4.10 Viability improvement of cortical neurons after 24 h of coculture with cortical astrocytes previously challenged with GA and rescued with glutamate.

Following the confirmation of the damage caused by GA in neurons cocultured with astrocytes, the role of glutamate in regulating neuronal metabolism and intercellular communication was evaluated. Notably, the addition of glutamate after 24 (Fig. 15A) and 40 hours (Fig. 15B) of GA exposure in primary rat cortical astrocytes induced positive effects by significantly ameliorating neuronal viability during the 24 h coculture.

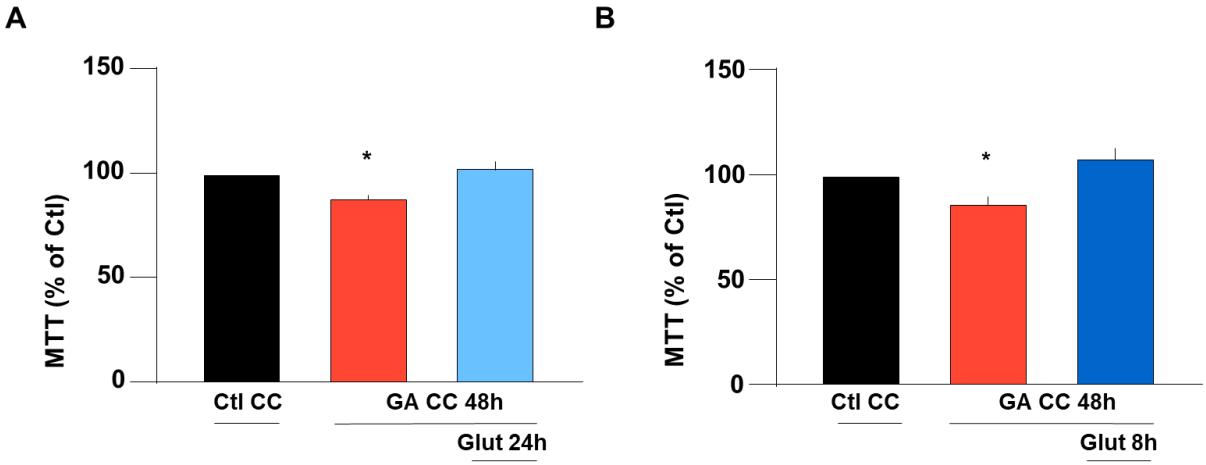
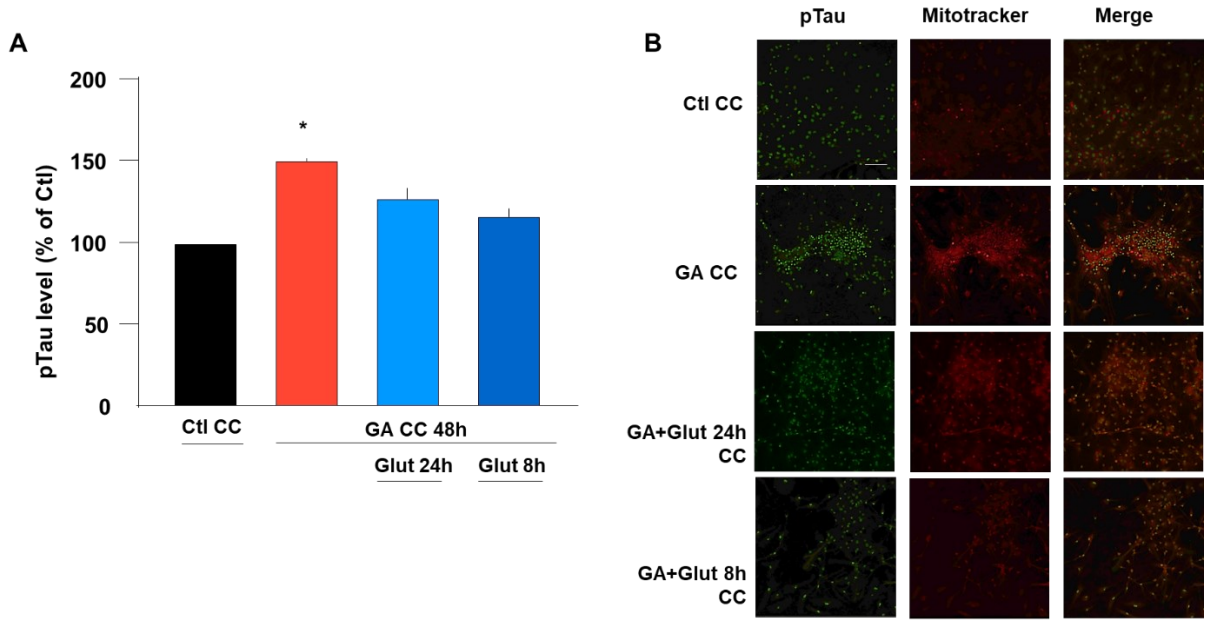


Figure 15: Protective effect of glutamate on cortical neurons viability after 24 h of coculture with cortical astrocytes previously challenged with GA. Glutamate was added after 24 h (A) and 40 h (B) of GA treatment for 48 h on cortical astrocytes, assessed by MTT assay. Cell viability results are graphed the column charts and reported as percentages relative to the respective control groups. Each column represents the mean ± S.E.M. of at least 3 experiments performed in triplicate. Significant differences were evaluated by one-way ANOVA followed by Dunnett's post hoc test. A $F(2, 24) = 9{,}192$. * Significant vs all groups ($p < 0.01$ vs CTL, $p < 0.001$ vs GA48h+Glut24h. B $F(2, 18) = 7{,}711$. * Significant vs all groups ($p < 0.05$ vs CTL, $p < 0.01$ vs GA48h+Glut8h).

4.11 Decrease in AD biomarker levels in cortical neurons after 24 h of coculture with cortical astrocytes previously challenged with GA and rescued with glutamate.

As previously reported, cortical neurons subjected to the coculture with cortical astrocytes previously challenged with GA exhibited a reduction in cell viability, which was paralleled by a notable elevation in both extracellular Aβ and intracellular pTau levels. Since glutamate was able to ameliorate cortical neurons viability cocultured with cortical astrocytes, it was of interest to determine whether glutamate's neuroprotective characteristics would also yield a beneficial effect on AD biomarker levels. Significantly, the investigations showed that glutamate added after 24 and 40 h of GA exposure in primary rat cortical astrocytes, there was a substantial decrease in the cellular accumulation of both pTau (Fig. 16A, B) and Aβ (Fig. 16C, D).



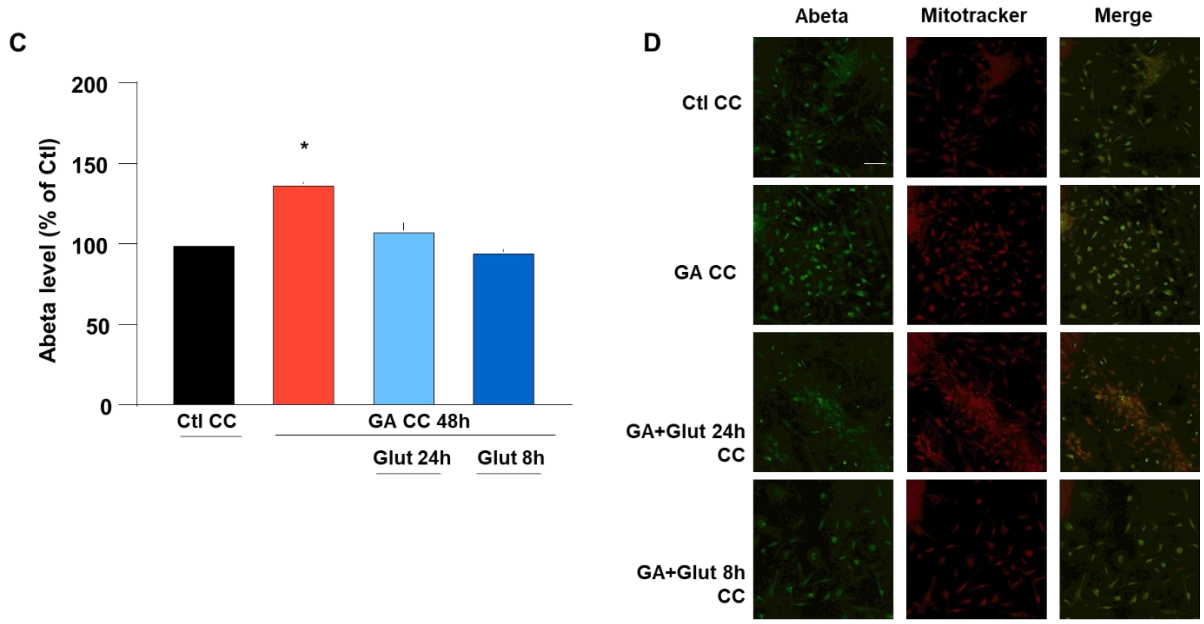


Figure 16: Effect of glutamate on pTau and Aβ expression in primary rat cortical neurons after 24 h of coculture with primary rat cortical astrocytes previously challenged with GA. Quantification (A, C) and representative images (B, D) of pTau and Aβ expression, respectively, on primary rat cortical neurons after 24 h of coculture with primary rat cortical astrocytes previously challenged with GA and Glutamate. Glutamate was added after 24 h (A) and 40 h (C) of GA treatment for 48 h on primary rat cortical astrocytes. The proteins pTau and Aβ were detected by immunofluorescence staining. In each experiment both the intensity of fluorescence of pTau and Aβ were reported as control percentages. Scale bar 50 μm. Each column represents the mean ± S.E.M. of at least 3 experiments performed in triplicate. Significant differences were evaluated by one-way ANOVA followed by Dunnett's post hoc test. AF (3, 12) = 27,42. * Significant vs all groups ($p < 0.0001$ vs CTL; $p < 0.01$ vs GA48h+Glut24h; $p < 0.001$ vs GA48h+Glut8h). B F (3, 16) = 54,45. * Significant vs all groups ($p < 0.0001$ vs all groups).

5 Discussion

A condition of hypometabolism in the brain it is known to cause mitochondrial dysfunction, energetic impairment, and oxidative stress, which are well known instances involved in the pathogenesis of neurodegenerative disorders, including AD. Converging evidence suggests that such condition might take place also in astrocytes, which exert a fundamental neuro-supportive role, making them main determinants of the degeneration of the surrounding neurons. The aim of this study was to explore the role of astrocytes in driving a cascade of detrimental effects in neurons and to investigate a possible approach able to limit the injury in both cell types. In particular, the main stressful stimulus that has been considered here was the metabolic impairment induced by GA in primary rat cortical astrocytes. In contrast to typical astrocytes, reactive astrocytes, which represent a hallmark of neuroinflammation during AD, downregulate supportive functions and begin to secrete neurotoxic factors leading to Aβ accumulation and toxicity also in the neighboring neurons [110]. The results obtained showed that, in primary rat cortical astrocytes, GA treatment resulted in 1) reduced cell viability, 2) oxidative stress, 3) neuroinflammation and 4) energetic failure. Moreover, metabolically impaired astrocytes contribute to the demise of the neighboring neurons in terms of 1) decrease in survival and 2) increase in pTau and Aβ levels. The main finding of the present work was that the enhancement of astrocytes energy metabolism, by supplying glutamate as an alternative metabolic substrate, improved the viability of both cortical astrocytes and neighboring neurons.

Recently, different studies showed that in neurons GA is able to create a condition of hypometabolism, which is accompanied by mitochondrial dysfunction and redox imbalance [100,101,103,111,112]. It is relevant to note that these experimental conditions effectively replicate the environmental factors frequently observed in the prodromal stages of AD.

Oxidative stress is a significant contributor to astrocyte-related inflammation and the progression of astrogliosis [113]. Under these specific conditions astrocytes become activated and respond by producing ROS within mitochondrial compartment [70]. Consistent with this evidence, in our experimental setting, 48 h exposure of GA caused a significant increase in mitochondrial ROS levels in cortical astrocytes. This oxidative status might be considered a main upstream event leading to astrocytes injury, affecting their viability, and provoking inflammatory responses. It is known that during neuroinflammation, increased concentrations of ROS cause the activation of NF-kB a critical transcription factor intimately associated with the control of immune and inflammatory signaling pathways [114]. In agreement with this evidence, the induction of metabolic stress through the use of GA has been observed to elicit a potent inflammatory response within cortical astrocytes, as observed by an increase in pNF-κB. Notably, this proinflammatory response culminates in a significant rise in the levels of TNF-α, a prominent cytokine, within the culture medium after 48 h of GA treatment.

Under different pathological conditions of hypometabolism, energy utilization can shift from glucose consumption to other substrates in both astrocytes and neurons [115]. In this regard, recent studies demonstrated that, in different neuropathological settings, glutamate can act as an alternative metabolic substrate by refilling oxidative metabolism thus preserving mitochondrial functions and ameliorating cell viability [116]. Considering the survival and energizing role of glutamate in neuropathology, in the present study the metabolic role of glutamate was investigated in cortical astrocytes challenged with GA. Interestingly, glutamate exposure protected against GA-neurotoxicity by promoting the improvement of cellular bioenergetics. In particular, the results obtained indicated that, under physiological conditions, the administration of glutamate stimulated the increase in ATP synthesis and

rescued the reduction in ATP production in cortical astrocytes challenged with GA for 48 hours. This observation underscores the pivotal role of glutamate, a vital neurotransmitter in the central nervous system, in supporting and maintaining the health of these glial cells [60,117]. In astrocytes, glutamate is taken up by two main transporters, namely GLT-1 and GLAST. In the light of the beneficial effects that glutamate exerted on the metabolic impaired astrocytes, the expression of the two transporters was explored. Interestingly, GLT-1 and GLAST were significantly upregulated after GA challenge, suggesting the induction of a compensatory response aimed at supporting glutamate enter to accomplish both energy needs and gliotransmitters balance. Therefore, it is possible to speculate that in cortical astrocytes, the metabolic alteration induced by GA might be mirrored by the increase in the two main glial glutamate transporters. In literature, it has been noted that defects involving glutamate transporters may be implicated in glutamate-induced neurodegenerative diseases, such as amyotrophic lateral sclerosis, and central nervous system damage [94]. Moreover, the correlation between neural activity and the expression of glutamate transporters GLT-1 and GLAST implies the presence of a feedback control mechanism that regulates glutamate balance within the brain. Disrupting this connection may potentially induce or worsen neuronal degeneration [93,118]. Indeed, defects in glutamate neurotransmission might also reflect a metabolic imbalance, considering the dual role of this molecule. The protective effect of glutamate is further substantiated by the findings derived from the analysis of mitochondrial ROS production and the assessment of pNF-κB expression and TNFα levels. Specifically, data elucidate that glutamate exerts protective effects GA-mediated neurotoxicity by reducing significantly mitochondrial ROS levels, expression of pNF-κB and TNFα level in cortical astrocytes treated with GA, further supporting its role in mitigating oxidative stress and, consequently, the neuroinflammation within these cells. The

exact underlying mechanisms deserve further investigations, however, starting from these results, the focus of the present work moved on investigating how the metabolic dysfunction in astrocytes may influence the neighboring neurons. The role of astrocytes in neuroinflammation has been highlighted in the past years, with many observations both in vivo and in vitro depicting the importance of these glial cells in this process [60]. The release of pro-inflammatory agents may induce cellular damage or even stimulate the production of Aβ in astrocytes, which in turn may affect redox balance, with a negative impact on the regulatory functions of astrocytes towards neurons [60]. Although astrocytes are not electrically excitable cells, they are essential for communication between astrocytes and neurons in the brain and also for maintaining neuronal metabolism. The use of the coculture system between cortical astrocytes and neurons allowed to assess the ability of astrocytes to affect the surrounding environment, thereby inducing a response in the neighboring neurons. For these reasons, neuronal functions were analyzed after 24 hours of coculture with astrocytes injured by GA for 48 hours. The results obtained demonstrated that metabolically impaired astrocytes compromised the homeostasis of the neighboring neurons in terms of viability, with an increase of both pTau and Aβ. Of particular interest is that the protective effects of glutamate observed on astrocytes significantly affected the surrounding neuronal environment. Specifically, a significant reduction in the two AD markers, pTau and Aβ was observed in neurons cocultured for 24 h with astrocytes challenged with 48 h of GA. Under physiological conditions, glutamate is released by astrocytes in response to various signals, such as neuronal stimulation or changes in environmental conditions [119]. Once released, glutamate serves several functions including neural communication and the glutamate-glutamine cycle [117]. This cycle is crucial for regulating the extracellular glutamate concentration and supporting neuronal metabolism [120].

In conclusion, in this study it has been demonstrated that the cytotoxicity induced by GA may trigger a cascade of events in astrocytes, including reduced viability, increased ROS, elevated release and expression of pro-inflammatory molecules., This astrocytic damage adversely affected neighboring cells, with main reference for neurons. The resulting neuronal damage involved reduced viability and increased AD marker levels. Ultimately, glutamate, used as an alternative metabolic substrate to mitigate astrocytic injury, exhibited a protective effect on both GA-treated astrocytes and neurons cocultured with astrocytes. A growing consensus emerges regarding the profound implications of mitochondrial dysfunction and metabolic impairment on astrocytes and the neighboring neurons they support. Moreover, the emerging prospects of using alternative metabolic substrates to glucose to counteract the hypometabolic state observed in the initial phases of AD reveal an increasingly optimistic avenue. This exploration of alternative metabolic pathways offers a glimpse of hope in addressing the fundamental metabolic alterations seen in the early progression of AD. Better understanding of the metabolic crosstalk between astrocytes and neurons could lead to new therapeutic strategies to slow or retard AD progression, and thereby improve the quality of life for millions of people worldwide.

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