Biomedicine & Pharmacotherapy 199 (2026) 119473

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Glyceraldehyde-induced metabolic defects in cortical astrocytes: implication for Alzheimer's disease pathogenesis and therapy

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ARTICLE INFO

Keywords:
Alzheimer's disease
glutamate
bioenergetics
cortical astrocytes
inflammation
autophagy impairment
Ca^{2+} dysfunction

ABSTRACT

A growing body of evidence suggests that reduced metabolic activity in astrocytes may compromise their normal supportive role for neurons and trigger pathophysiological pathways that contribute to the progression of Alzheimer's disease (AD). Due to the complexity of AD pathophysiology, it is crucial to study the disease not only within those contexts traditionally viewed from a neuron-centric perspective. In this study, we settled up a new model of AD by exposing primary rat cortical astrocytes to glyceraldehyde (GA), an inhibitor of glycolytic pathway able to induce a significant hypometabolism and recapitulate several AD pathomechanisms. Accordingly, GA-induced hypometabolism produced (a) astrocytosis, as revealed by the increase in GFAP and Glutamine Synthase (GS) immunosignals, (b) mitochondrial dysfunction, detected as reduced ATP level, mitochondrial ROS hyperproduction and Ca^{2+} dyshomeostasis at both cytosolic and mitochondrial level (c) inflammation, measured as NF-κB activation, TNFα release, AGEs hyperproduction/RAGE hyperexpression and increase in S100β immunosignal, and, finally (d) autophagy impairment, characterized by the p62 and LC3II protein accumulation. By virtue of glutamate ability to stimulate cell metabolism, we examined the effect of the neurotransmitter supplementation on cell damage and those correlated mechanisms in the proposed AD model. Of interest, metabolic, inflammatory and autophagy defects were mitigated when astrocytes were exposed to glutamate as metabolic boosting substrate. The protective effect of glutamate was counteracted by the pharmacological inhibition of astrocytic glutamate transporters, thus highlighting the relevance of glutamate intracellular action. Collectively, these results highlight the importance of considering astrocyte-targeted therapies as potential strategy in AD.

1. Introduction

Alzheimer disease (AD), a multifactorial non-cell autonomous neurodegenerative disease, is considered as an unmet medical need lacking of disease-modifying drugs. The multifactorial hypothesis on its pathogenesis emphasizes the concomitant action of multiple dysfunctional processes occurring in several brain areas that include calcium (Ca^{2+}) dyshomeostasis, deregulation of quality protein control, overactive neuroinflammation, impaired mitochondria and oxidative damage, senile plaques and neurofibrillary tangles deposition, synapse degeneration, reactive astrocytes proliferation, and alteration of the microglial phenotype. Moreover, the breaking down of metabolism is one of the major critical events shared by all the cells involved in AD progression and preceding most of these pathomechanisms. In consideration of the high energy demand by active neurons, astrocytes have been considered as the major source of neuronal energy that provides these cells with lactic acid produced by aerobic glycolysis. Several pieces of evidence agree that most of the brain glycolysis mainly occurs in

Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; AGE, advanced glycation end-product; RAGE, receptor of AGE; ATP, adenosine triphosphate; Bafilo, Bafilomycin; BCH, 2-Aminobicyclo- 2,2,1-heptane-2-carboxylic acid; Ca^{2+}, calcium; EAATs, excitatory amino acid transporters; GA, glyceraldehyde; GDH, glutamate dehydrogenase; Glut, glutamate; GFAP, glial fibrillary acidic protein; GS, Glutamine Synthase; LC3, microtubule-associated protein 1 A/1B-light chain 3; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; pTau, phosphorylated Tau; ROS, reactive oxygen species; S100β, S100 Ca^{2+}-binding protein B; TNF-α, tumor necrosis factor α.

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https://doi.org/10.1016/j.biopha.2026.119473
Received 18 December 2025; Received in revised form 22 April 2026; Accepted 28 April 2026
Available online 1 May 2026

astrocytes [1–3]. Furthermore, there are strong evidence that astrocytic glycogen plays an indispensable role in physiological brain activity [4,5] supporting the increase of neuronal activity induced by several stimuli [6].

Therefore, astrocytes are essential regulators of the central nervous system homeostasis and play a vital role in supporting neuronal health [7–12]. However, these cells exert a dual role in astrocyte-neuron communication since "healthy" astrocytes promote neuronal survival, while "injured" astrocytes release harmful neurotoxins that negatively affect neuronal viability [13,14].

In AD, astrocytes can become dysfunctional and fail to provide adequate metabolic support to neurons, impacting their energy balance, and increasing neuronal vulnerability, thus contributing to the disease progression [15]. However, pathological mechanisms in AD astrocytes are not fully understood but their knowledge should help in the identification of new druggable targets.

In recent years, there has been an increasing focus on the role of Advanced Glycation End Products (AGEs) in driving and/or worsening metabolic dysregulation [16–18]. AGEs are byproducts of abnormal glucose and fructose metabolism, especially when an excess of glyceraldehyde (GA) accumulates in the cellular environment. These compounds are known to cause oxidative stress, trigger inflammation impairing synaptic function. Recent studies have demonstrated that exposure to GA in primary rat cortical neurons, by inhibiting the glycolytic pathway, may induce a hypometabolic state that mirrors the key pathological features of AD [19–22]. The decrease in neuron viability caused by GA is linked to redox oxidative status disruption, mitochondrial dysfunction, perturbations in Ca^{2+} homeostasis, elevated levels of AD biomarkers, and alterations in the AMPK-mTOR signaling pathway. Significantly, boosting energy metabolism and antioxidant activity can alleviate the toxicity induced by GA in neuronal cells [19–22].

Moreover, astrocytes express the receptor for AGEs named RAGE, which is able to mediate the action of several endogenous molecules released either under physiological stress or chronic inflammation [23]. Besides AGEs, RAGE can also bind S100 Ca^{2+}-binding protein B (S100β), amyloid-β (Aβ) and other additional molecules thus activating the nuclear factor kappa B (NF-κB) transcription pathway and enhancing the inflammatory cascade. Of interest the inhibition of RAGE/NF-κB signaling pathway in astrocytes has been proposed as new therapeutic strategy for the treatment of neurodegenerative disorders [23]. However, during neurodegeneration astrocytic inflammation has been linked to other mechanisms including autophagy, able to amplify disease's progression [24]. Specifically, defective lysosomal function may impair astrocytic autophagy determining accumulation of toxic protein aggregates and propagation of neuroinflammation [24].

In this respect, new evidence has indicated that impaired autophagy contributes to AD pathogenesis and progression [25]. In fact, Aβ is preferentially degraded by astrocytes [26,27], via autophagy pathway mediated by microtubule-associated protein 1 A/1B-light chain 3-II (LC3II) and p62 that could be properly activated in response to Aβ oligomer [28]. This mechanism may confer a strong resilience to the active astrocytes involved in the early phase of the disease [29]. Accordingly, astrocyte-specific knockdown of LC3II and p62 significantly increases Aβ plaque formation and astrogliosis in AD transgenic mice, along with their cognitive function decline. However, autophagy hyperactivation determines significant metabolic alterations in severe proliferative reactive astrocytes [30], a condition considered to be sufficient for neurodegeneration in AD mice [28]. Of note, LC3II and p62 proteins have been found concomitantly increased in hippocampal astrocytes of AD mice [31] thus underlying a detrimental role of astrocytic autophagy engulfment as a pathomechanism of the disease. Very interestingly, it has been demonstrated that glutamate may induce autophagic flux in astrocyte in a dose- and time- dependent manner [32]. However, the effects on autophagy and its putative correlation with the metabolic action of glutamate remain elusive. Moreover, the mechanisms underlying glutamate-induced modulation of autophagy and metabolic pathways are not deeply explored in AD astrocytes thus pushing our research into the identification of new therapeutic targets in AD astrocytes. Therefore, the use of glutamate to fuel astrocyte metabolism and autophagy together with the relative mechanisms have been explored in this astrocytic model of AD.

2. Results

2.1. Effect of GA on cell viability, Ca^{2+} homeostasis, mitochondrial ROS formation, ATP production and inflammatory markers in cortical astrocytes

To induce a hypometabolic state, rat cortical astrocytes were exposed to the glycolysis inhibitor GA (1 mM) [19–22]. We initially evaluated the impact of GA on cell viability by treating cells for 8, 16, 24, and 48 h. The results showed that GA cytotoxicity started to be significant after 24 h of treatment with even greater toxicity observed at 48 h, as measured by the significant reduction in mitochondrial activity (Fig. 1 A) and enhanced lactate dehydrogenase (LDH) release (Fig. 1 B). To establish a more reliable and reproducible model, we selected the GA 48 h treatment for all subsequent experiments. Astrocytes exert a crucial role in providing neurotrophic support, regulating metabolism and maintaining redox homeostasis. However, they can undergo reactive changes in response to various stimuli. To assess astrocyte activation, we measured the levels of glial fibrillary acidic protein (GFAP). Our results revealed a significant increase in GFAP immunosignal after GA treatment (Fig. 1 C a,b), indicating that GA exposure may induce a shift from the resting to the reactive state in astrocytes. Previous studies demonstrated that GA negatively affected cellular bioenergetics and redox state in cortical neurons [19–22]. Hence, to better elucidate the mechanisms underlying astrocytes death and the involvement of mitochondrial suffering, we investigated the effect of GA on intracellular Ca^{2+} homeostasis, ATP synthesis and mitochondrial ROS production. Considering the influence of GA exposure on Ca^{2+} homeostasis in neuronal cells [11,13,14], our findings revealed an increase in the basal level of intracellular Ca^{2+} within both cytosolic ($[Ca^{2+}]_i$) and mitochondrial Ca^{2+} compartment ($[Ca^{2+}]_{mit}$) following 48 h of GA treatment (Fig. 1 D, E). Furthermore, a significant decrease in intracellular ATP levels (Fig. 1 F) and a greater increase in mitochondrial ROS production (Fig. 1 G a,b) were detected in cortical astrocytes following exposure to GA. Considering that GA treatment stimulated the astrocytes activation along with mitochondrial dysfunction and oxidative stress, we further explored whether the cytotoxic effect of GA could also trigger an inflammatory response in cortical astrocytes. We found a significant activation of the nuclear factor-κB (NF-κB), measured as p65 phosphorylation (Fig. 1 H a, b) and a relevant elevation in the levels of the pro-inflammatory cytokine tumor necrosis factor α (TNF-α) (Fig. 1 I).

2.2. Effect of glutamate on GA-induced astrocytosis

After assessing the cytotoxicity of GA on cortical astrocytes, we proceeded to explore whether stimulating energy metabolism by glutamate, as an alternative metabolic substrate, could lead to a restoration of cell viability and metabolic state. Since cell viability started to be significantly compromised after 24 h of GA treatment, we supplemented the culture medium with glutamate in the last 24 h of GA exposure. The results showed that GA cytotoxicity was significantly cushioned by glutamate as measured by the enhanced mitochondrial activity (Fig. 2 A) and reduced LDH release (Fig. 2 B). Concerning the range of concentrations in which glutamate may act as survival factor in GA model, it produced positive effect on mitochondrial activity in the narrow concentration range of 0.3–3 mM in cortical astrocytes, while it was not protective starting from 10 mM (Supplementary figure S1 A). Then, the role of glutamate on GA-induced astrocyte activation was examined. When astrocytes were supplemented with glutamate during

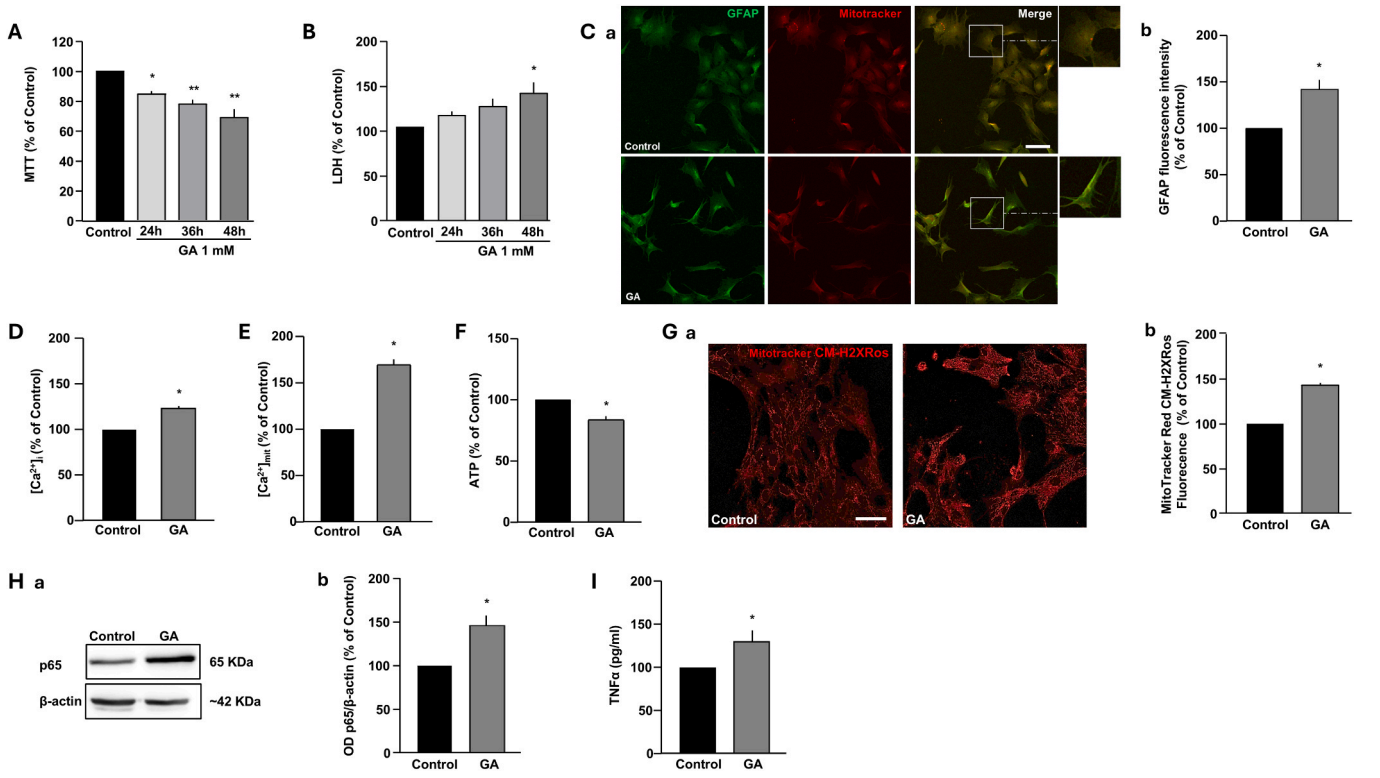

Fig. 1. Effect of glyceraldehyde (GA) on cortical astrocytes' survival and function. Time-dependent effect of GA on mitochondrial activity measured as MTT(A) and LDH release (B). Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs respective controls; **p < 0.05 vs previous time points. (C) GFAP immunosignal and Mitotracker pictures as single or merged images under control conditions and after GA exposure (a) and quantification (b). Scale bar 50 μM. Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs control. (D) Intracellular and (E) mitochondrial Ca^{2+} level quantifications under control conditions and after GA exposure (each bar represents the mean of 30–50 single cells recorded in different experimental sessions). Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs control. (F) ATP level under control and after GA exposure. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control; (G) Mitotracker CM-H2XRos images (a) and quantification (b) under control conditions and after GA exposure. Scale bar 50 μM. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control. (H) Representative Western blotting (a) and quantification (b) of p65 subunit under control and after GA exposure. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control. (I) Graph bar depicting TNFα level quantification under control and after GA exposure. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control.

the final 24 h of GA treatment, we observed a significant reduction of GFAP- (Fig. 2 C a,b) and glutamine synthetase (GS)- immunosignals (Fig. 2 D a,b).

Targeting S100β may be useful in AD therapy, being this protein involved in the pathogenesis of the disease [33,34]. Predominantly secreted by astrocytes, S100β could be released in response to intracellular Ca^{2+} fluctuations [35]. Of note, under these experimental conditions mimicking AD astrocytosis, GA significantly increased S100β immunosignal that was consistently reduced by glutamate (Fig. 2 E a,b). This further highlights that, at higher expression level, S100β may contribute to neuronal damage and neuroinflammation.

2.3. *Effect of glutamate on GA-induced mitochondrial damage, inflammation and Ca^{2+} dyshomeostasis in cortical astrocytes*

Considering the boosting effect of glutamate on cell metabolism and the consequent protective effect on astrocytes exposed to GA and, with the aim to better clarify the underlined mechanism, the effect of glutamate on $[Ca^{2+}]_i$ and $[Ca^{2+}]_{mit}$, ATP, mitochondrial ROS production, and some inflammatory markers were investigated.

When added during GA challenge, glutamate significantly restored $[Ca^{2+}]_i$ and $[Ca^{2+}]_{mit}$ (Fig. 3 A, B) as well as the intracellular levels of ATP (Fig. 3 C). Considering also its antioxidant role in different pathological settings [19,36], glutamate effectively reduced mitochondrial ROS production which was significantly compromised after GA challenge (Fig. 3 D a,b).

As GA triggered an inflammatory response in cortical astrocytes, we also investigated the impact of glutamate on this inflammatory state. Our findings revealed that the addition of glutamate after 24 h of GA treatment mitigated the GA-induced inflammatory response by decreasing both the expression of p65 (Fig. 3 E) and release of the pro-inflammatory cytokine TNF-α (Fig. 3 F). To validate GA-induced degeneration as a disease-specific model, phosphorylated Tau (pTau) has been measured in astrocytes exposed to GA. Fig. 3 showed a significant accumulation of pTau in astrocytes (Fig. 3 G a) that was reduced in the presence of the allosteric activator of glutamate dehydrogenase (GDH) 2-Aminobicyclo- (2,2,1)-heptane-2-carboxylic acid (BCH) (Fig. 3 G a,b).

2.4. *Role of glutamate transporters in cortical astrocytes exposed to GA*

The controlled regulation of glutamate homeostasis allows astrocytes to perform crucial function in the central nervous system [37]. Among the primary regulators of extracellular glutamate, the excitatory amino acid transporters EAAT1 (also known in rodent as a glutamate-aspartate transporter, GLAST) and EAAT2 (also known in rodent as a glutamate transporter-1, GLT-1) are predominantly expressed in astrocytes. These astrocytic transporters are responsible for clearing excess glutamate from the synaptic cleft to optimize neuronal functions and prevent glutamate excitotoxicity [38]. Therefore, with the aim to study the impact of GA on the regulation of glutamate homeostasis in astrocytes, we assessed the expression of these transporters following 48 h of

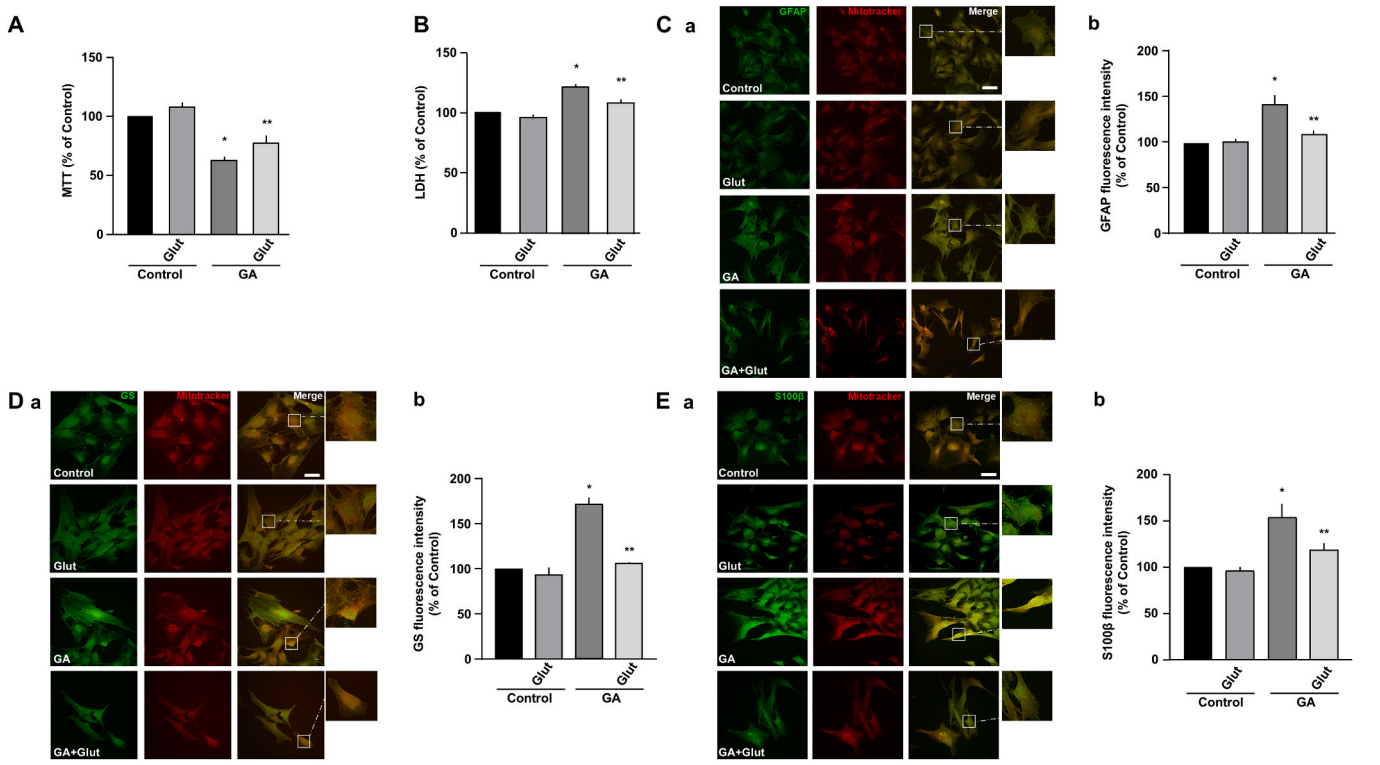

Fig. 2. Effect of glutamate supplementation on GA induced astrocytes' activation. Quantification of glutamate supplementation (to the last 24 h of GA treatment) on mitochondrial activity measured as MTT(A) and LDH release (B). Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs respective controls, **p < 0.05 vs GA alone. (C) GFAP immunosignal and Mitotracker pictures as single or merged images under control conditions and after GA exposure in absence or presence of glutamate (a) and quantification as fluorescence intensity (b). Scale bar 50 μM. Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs controls, **p < 0.05 vs GA alone. (D) GS immunosignal and Mitotracker pictures as single or merged images under control conditions and after GA exposure in absence or presence of glutamate (a) and quantification as fluorescence intensity (b). Scale bar 50 μM. Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs controls, **p < 0.05 vs GA alone. (E) S100β immunosignal and Mitotracker pictures as single or merged images under control conditions and after GA exposure in absence or presence of glutamate (a) and quantification as fluorescence intensity (b). Scale bar 50 μM. Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs controls, **p < 0.05 vs GA alone.

exposure to GA and observed a significant elevation in both GLT-1 and GLAST protein expression (Fig. 4 A a,b) and immunosignals (Fig. 4 A c).

To establish the modality and the site of action of glutamate in exerting its protective effects, viability parameters were studied by supplementing glutamate alone or in the presence of the unspecific transporter inhibitor (3*S*)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino] phenyl]methoxy]-L-aspartic acid TFB-TBOA (TFB, 1 μM). The pharmacological inhibition of GLT-1 and GLAST by TFB prevented protective effects of glutamate on mitochondrial activity and LDH release (Fig. 4 B, C) thus highlighting the necessity of glutamate to penetrate into the cell in order to exert its metabolic boosting effects. Of interest, at 30 μM the unspecific transporters' inhibitor TFB exacerbated the detrimental effect of GA in astrocytes, thus demonstrating the importance of EAAT increased expression as protective factor (Supplementary Figure S1 B). Furthermore, the effects of GA on the expression of RAGEs and their proinflammatory ligands AGEs were also reverted by glutamate (Fig. 4 D, E) and these events were prevented by TFB (Fig. 4 D, E), thus demonstrating the relevance of intracellular glutamate site to counteract inflammation in AD models. Accordingly, TFB reverted the effect of glutamate on GA-induced NF-κB activation in cortical astrocytes (Fig. 4 F).

2.5. Glutamate-induced recovery of autophagy impairment in cortical astrocytes exposed to GA

Considering the detrimental effect of GA on astrocytes' function and the determinant role of autophagy impairment in the protein quality control under neurodegenerative conditions, the impact of hypometabolism on autophagy has been investigated under the same experimental conditions. Of note, after exposure to GA a significant increase in the phosphorylation/activation of the autophagy initiator AMPK has been detected in cortical astrocytes (Fig. 5 A, B). Moreover, also a consistent upregulation of both p62 and LC3II protein expression was measured (Fig. 5 A, C, D), as sign of autophagy impairment. Of interest, supplementing cortical astrocytes with glutamate, pAMPK, p62- and LC3II- expression returned to their basal levels (Fig. 5 A-D), suggesting a restored autophagy in astrocytes. However, as occurred for inflammatory marker expression, supplementing glutamate in the presence of the unspecific transporters' inhibitor TFB counteracted glutamate-induced restoring effects on autophagy (Fig. 5 A-D). Of note, Lysosomal Inhibition Assays performed by V-ATPasi inhibitor bafilomycin A1 (Bafilo) on LC3 expression showed that GA induced a partial autophagy flux blockade (Fig. 5 E a-c).

3. Discussion

Brain glucose hypometabolism is known to cause mitochondrial dysfunction, energy deficits, and oxidative stress, all key factors in the early phase of AD pathogenesis [12,39]. Accumulating evidence suggests that similar conditions occur also in astrocytes, compromising their physiological neuro-supportive functions and contributing to the degeneration of the surrounding neurons [40,41]. In fact, during AD, astrocytes undergo a functional decline characterized by altered morphology and reduced glucose metabolism [8,42]. Of note, this astrocytic metabolic dysfunction is increasingly recognized as a key factor driving Aβ deposition and toxicity in the neighboring neurons

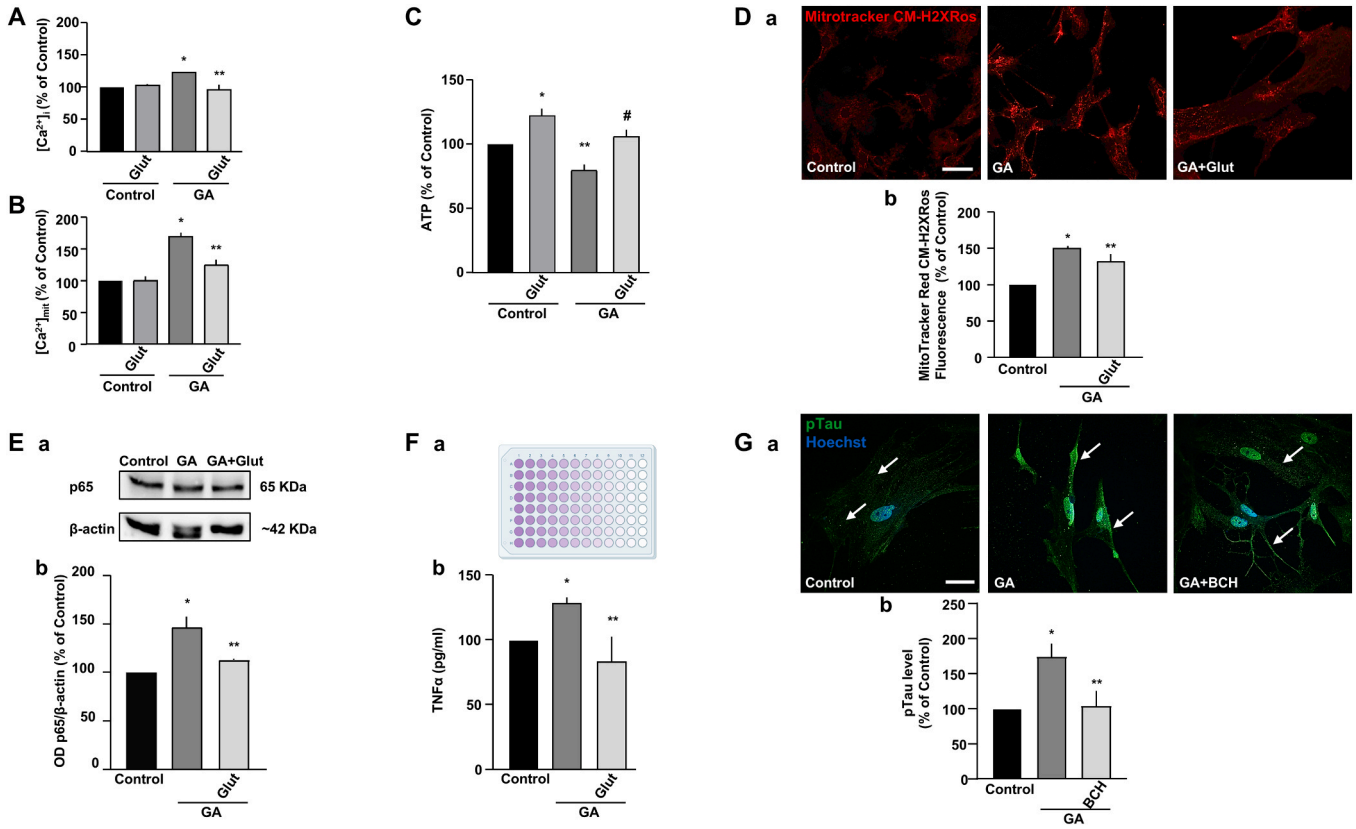

Fig. 3. Effect of glutamate supplementation on GA induced intracellular Ca^{2+} dyshomeostasis and inflammation in cortical astrocytes. (A-B) Quantification of glutamate supplementation (to the last 24 h of GA treatment) on intracellular ($[Ca^{2+}]_i$) and mitochondrial Ca^{2+} levels ($[Ca^{2+}]_{mit}$) measured in single-cells (N = 30 for each experimental group). Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs control, **p < 0.05 vs GA alone. (C) Quantification of ATP level under control conditions and after GA exposure in absence or presence of glutamate. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control, **p < 0.05 vs control groups, p < 0.05 vs GA alone. (D) Mitotracker CM-H2XRos images (a) and quantification (b) under control conditions and after GA exposure in absence or presence of glutamate. Scale bar 50 μM. Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs control, **p < 0.05 vs GA alone. (E) Representative Western blotting (a) and quantification (b) of p65 subunit under control and after GA exposure in absence or presence of glutamate. Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs control, **p < 0.05 vs GA alone. (F) Graph bar depicting TNFα level quantification under control conditions and after GA exposure in absence or presence of glutamate. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control, **p < 0.05 vs GA alone. (G) pTau immunosignal and Hoechst pictures as single or merged images under control conditions and after GA exposure in absence or presence of BCH (a) and quantification as fluorescence intensity (b). Arrows indicates specific accumulation of pTau. Scale bar 50 μM. Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs controls, **p < 0.05 vs GA alone.

during neurodegeneration [43–46]. In this study, as previously observed in cortical neurons [19–22], we found that the hypometabolic state induced by glyceraldehyde (GA) in cortical astrocytes led to: (I) pTau accumulation, (II) decreased cell viability, (III) energy depletion, (IV) oxidative stress, (V) inflammation, (VI) disruption of Ca^{2+} homeostasis, and (VII) autophagy impairment. This detrimental pattern clearly stated that GA exposure mimics the hypometabolism observed in AD. Of interest, hypometabolism is known to drive the conversion of resting astrocytes into a reactive state, characterized by increased expression of the intermediate filament protein GFAP [47,48]. In our experimental model, we observed an increase in GFAP and GS expressions, along with intracellular ATP depletion and an overload of mitochondrial ROS. This aligns with previous studies showing elevated levels of GFAP during the mild cognitive impairment stage, as well as in cerebrospinal fluid samples from patients with AD and other dementias [49,50]. Similarly, a progressive hyperactivation of astrocytes and correlated increased GFAP levels in the rat hippocampus has also been reported following induction of a diabetic state through streptozotocin treatment [51]. Reactive astrogliosis is also closely associated with the upregulation of NF-κB pathway and increased production of pro-inflammatory cytokines such as TNF-α, contributing to reduced neuronal fitness and cognitive decline [52–54]. Transcriptome analyses of AD mouse models further revealed that astrocytes exhibit a downregulation of genes crucial for neuronal function alongside an upregulation of genes related to inflammation [55,56]. NF-κB is a key regulator in this process, acting as a critical sensor of metabolic imbalances and playing a pivotal role in the regulation of inflammatory signaling pathways [57–59]. However, the role of NF-κB in AD is complex and controversial: given its dual functions in promoting both pro-inflammatory and anti-inflammatory responses, its activity needs to be tightly regulated [60,61]. Here we found that GA treatment stimulated a considerable pro-inflammatory response in cortical astrocytes, marked by the activation of NF-κB and a significant increase in TNF-α levels, confirming that the hypometabolic state induced by GA may trigger the conversion of resting astrocytes into a reactive detrimental state. In the light of the present increase of GS immunosignal in cortical astrocytes exposed to GA, we hypothesize that this enzyme could be assured not only as a marker of astrocytes' reactive state but also as a sign of inflammation.

Another intriguing aspect of the prodromal stage of AD is the alteration of Ca^{2+} homeostasis in astrocytes [62,63]. It has been observed that an abnormal disruption of astrocyte Ca^{2+} signaling can impact cellular viability and impede their ability to regulate neuronal activity, potentially instigating or sustaining network hyperactivity in AD [9,15,64]. Consistent with these findings, we found that GA treatment

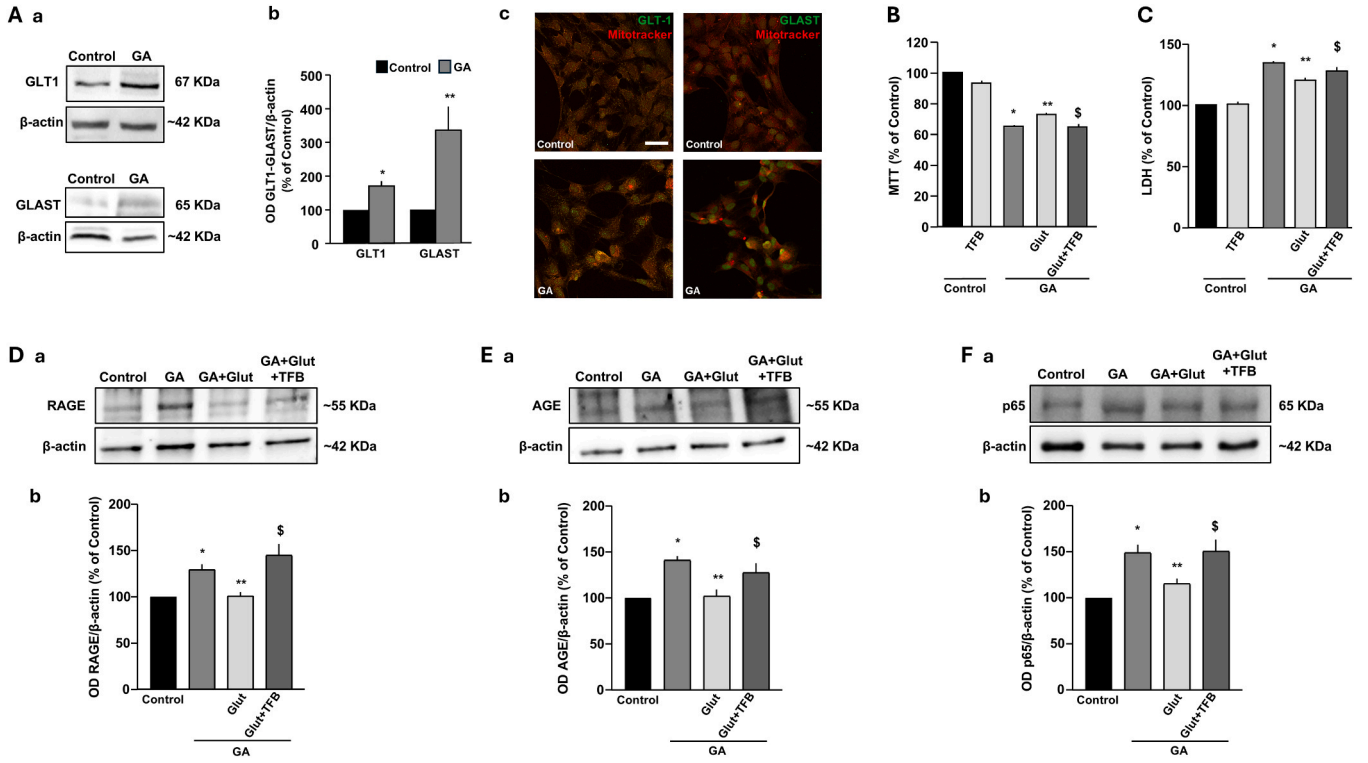

Fig. 4. Effect of glutamate transporters' inhibition on glutamate counteracting effects in cortical astrocytes exposed to GA. (A) Representative Western blotting (a) relative quantification (b) and immunosignals (c) of GLT-1 (up) and GLAST (bottom) in controls and after GA exposure (48 h). Scale bar 50 μM. Data are expressed as mean ± S.E.M. of three different experimental sessions. *p < 0.05 vs respective controls; **p < 0.05 vs all. (B) Quantification of the effect of the pharmacological inhibitor of glutamate transporters, TFB (1 μM), on glutamate -modulated mitochondrial activity measured as MTT. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs controls, **p < 0.05 vs GA alone, $p < 0.05 vs GA+Glut. (C) Quantification of the effect of the pharmacological inhibitor of glutamate transporters, TFB (1 μM), on glutamate-modulated LDH release; TFB was administered 1 h before the addition of glutamate. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs controls, **p < 0.05 vs GA alone, $p < 0.05 vs GA+Glut. (D) Representative Western blotting (a) and quantification (b) of RAGE expression under control conditions and after exposure to GA alone, GA in the presence of glutamate (GA+Glut) or GA in the presence of glutamate and TFB (GA+Glut+TFB); TFB was administered 1 h before the addition of glutamate. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control, **p < 0.05 vs GA alone, $p < 0.05 vs GA+Glut. (E) Representative Western blotting (a) and quantification (b) of AGE under control conditions and after exposure to GA alone, GA in the presence of glutamate (GA+Glut) or GA in the presence of glutamate and TFB (GA+Glut+TFB); TFB was administered 1 h before the addition of glutamate. *p < 0.05 vs control, **p < 0.05 vs GA alone, $p < 0.05 vs GA+Glut. (F) Representative Western blotting (a) and quantification (b) of p65 subunit under control conditions and after exposure to GA alone, GA in the presence of glutamate (GA+Glut) or GA in the presence of glutamate and TFB (GA+Glut+TFB); TFB was administered 1 h before the addition of glutamate. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control, **p < 0.05 vs GA alone, $p < 0.05 vs GA+Glut.

markedly increased both intracellular and mitochondrial Ca^{2+} basal levels in cortical astrocytes, suggesting that disturbances in Ca^{2+} homeostasis worsen the existing metabolic deficiencies and oxidative stress in these cells.

That a Ca^{2+}-dependent protein S100β was also increased by GA supports the hypothesis that intracellular and mitochondrial Ca^{2+} levels may control the inflammatory status of astrocytes, thus playing an upstream coordinating role in this detrimental process. Indeed, the metabolic impairment of astrocytes in the pathophysiology of AD has recently attracted considerable interest with an initial metabolic increase followed by a subsequent metabolic deficit. The significant increase in S100β and TNF-α levels we observed in astrocytes exposed to GA is consistent with previous findings reported in both *in vivo* models and human iPSC-derived astrocytes during astrocytic activation. In particular, Caceres-Palomo and colleagues [65] observed metabolic alterations in hiPSC-derived astrocytes from AD patients. These alterations were accompanied by a reactive phenotype, an increase in TNF-α levels and, interestingly, a significantly higher consumption of glutamate compared to iPSC-derived astrocytes from normal cognition individuals. Increased TNF-α levels have also been described in rats where AD was induced by intrahippocampal injection of $A\beta_{1-42}$ and astrocytic metabolism was chronically inhibited by fluorocitrate [66]. As for S100β, whose levels increase during astrocytic activation, a correlation with metabolic alterations has been described in a setting of early-stage AD patients [67].

Supposing that astrocytes with impaired metabolism could play a critical role in nearby neurons' deterioration thus constituting a therapeutical target against neurodegeneration, it appeared of interest to investigate the putative mechanisms and how the implementation of alternative energy sources could enhance astrocytes' metabolism. Disrupting astrocyte metabolism limits neuronal energy supply in virtue of Astrocyte-Neuron Lactate Shuttle (ANLS) hypothesis leading to bioenergetic failure, synapse loss, and accelerated neurodegeneration. Identifying new pathomechanisms at astrocytic level may be useful for neurodegeneration therapy. In this sense, although the absence of neuron- astrocyte interaction experiments could be considered as a limitation of the study, dissecting the contribution of astrocytes to this phenomenon has been useful to highlight their "non-cell autonomous" role.

Based on our previous findings [19,20,36], we delved into the impact of activating alternative pathways triggered by glutamate treatment. In fact, under conditions of energy deficiency, such as hypometabolism, cells may shift from using glucose to alternative energy sources in order to sustain their functions [68,69]. Recent findings have shown that, in various neuropathological contexts, providing these alternative metabolic substrates can support oxidative metabolism, preserve

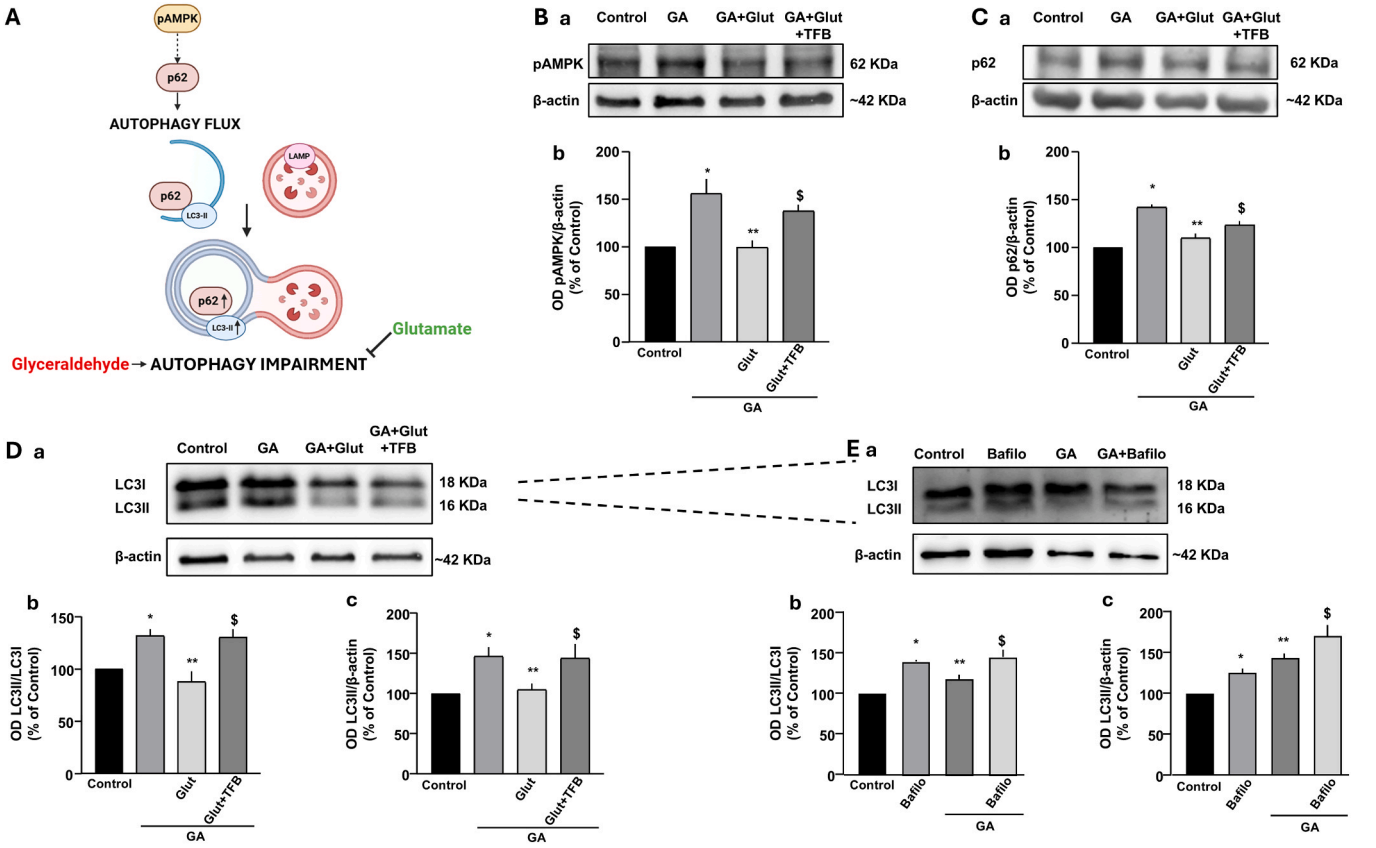

Fig. 5. Effect of glutamate and glutamate transporters' inhibition on autophagy impairment in cortical astrocytes exposed to GA. (A) Schematic cartoon depicting the effect of glutamate on GA-mediated autophagy defects. (B) Representative Western blotting (a) and quantification (b) of pAMPK expression under control conditions and after exposure to GA alone, GA in the presence of glutamate (GA+Glut) or GA in the presence of glutamate and TFB (GA+Glut+TFB); TFB was administered 1 h before the addition of glutamate. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control, **p < 0.05 vs GA alone, $p < 0.05 vs GA+Glut. (C) Representative Western blotting (a) and quantification (b) of p62 expression under control conditions and after exposure to GA alone, GA in the presence of glutamate (GA+Glut) or GA in the presence of glutamate and TFB (GA+Glut+TFB); TFB was administered 1 h before the addition of glutamate. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs control, **p < 0.05 vs GA alone, $p < 0.05 vs GA+Glut. (D) Representative Western blotting (a) quantification for its precursor (b) and quantification for β-actin (c) of LC3II expression under control conditions and after exposure to GA alone, GA in the presence of glutamate (GA+Glut) or GA in the presence of glutamate and TFB (GA+Glut+TFB); TFB was administered 1 h before the addition of glutamate. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. *p < 0.05 vs respective controls; **p < 0.05 vs GA alone, $p < 0.05vs GA+Glut. (E) Representative Western blotting (a) quantification for its precursor (b) and quantification for β-actin (c) of LC3II expression under control conditions and after exposure to GA alone, in the absence or in the presence of bafilomycin A1 (Bafilo, 100 nM); bafilomycin A1 was administered during the last hour of GA treatment. Data are expressed as mean ± S.E.M. of at least three different experimental sessions. b) *p < 0.05 vs control; **p < 0.05 vs control and control+Bafilo; $p < 0.05 vs GA. c) *p < 0.05 vs control; **p < 0.05 vs control; $p < 0.05 vs GA.

mitochondrial function, and enhance cell survival [19,20,36,70]. We interestingly observed that glutamate supplement -in a narrow concentration range- significantly improved the overall cellular functions of cortical astrocytes thereby counteracting those pathomechanisms elicited by GA-induced hypometabolism. Accordingly, glutamate protected cortical astrocytes against GA-cytotoxicity by recovering the reduction in ATP synthesis, thus ameliorating cell survival. This protective effect was reflected in reducing mitochondrial ROS production, GFAP and GS expression, pNF-κB activation, TNF-α levels, and normalizing Ca^{2+} levels within both the intracellular and mitochondrial compartments. Therefore, promoting astrocytes' oxidative metabolism can disrupt the harmful cycle initiated by GA, thereby preserving the health and function of astrocytes under stressful conditions. Similarly, in different models of AD, dietary supplements containing alternative metabolic sources, like ketone bodies, have demonstrated notable benefits [71–73]. These supplements enhance glycolysis and mitochondrial function, decreasing the accumulation of Aβ plaques and leading to improved cognitive performance. Furthermore, boosting endogenous glutamate recycling through increased activity of GDH activity has promoted ATP synthesis and ameliorated cell survival in conditions with severely compromised energy metabolism [74–78]. In accordance with the protective role of GDH activation in compromised neurons, the present data -showing the ability of its allosteric activation in reducing pTau accumulation in astrocytes exposed to GA- confirmed the positive role of glutamate as protective metabolic substrate also in AD astrocytes.

Furthermore, in the present study, new putative targets to facilitate the protective role of glutamate at astrocyte level during AD progression have been identified. For instance, the counteractive action of glutamate on the increased expression of GLT-1 and GLAST during GA-induced hypometabolism was proved. Moreover, in the light of the detrimental effect of hypometabolism on cortical astrocytes and in consideration of the protective effect mediated by glutamate supplementation, the upregulation of GLT-1 and GLAST induced by GA could be considered as a compensatory mechanism aimed at protecting astrocytes, which avidly tries to capture extracellular glutamate in order to boost cell metabolism. Accordingly, blocking the internalization of glutamate by an unspecific transporter inhibitor counteracted the protective effect of the neurotransmitter. Furthermore, at higher concentration, the unspecific blocker of these transporters exacerbated GA toxicity, thus confirming the supposed protective role of their upregulation.

Furthermore, the exact molecular mechanisms regulating glial glutamate transporters in AD are not fully understood. Indeed, various factors, including growth factors, cytokines, and alteration of cellular redox status are implicated in either positive or negative regulation of these transporters in different pathophysiological settings [79–84]. However, the NF-κB pathway is known to be involved in glutamate transporters regulation [80,83]. In this respect, it is plausible that a correlation might exist between the activation of the NF-κB pathway and the increase in both GLT-1 and GLAST induced by GA, since mutations in NF-κB binding site results in a reduced activity at EAAT1 promoter [80,85,86]. Therefore, we propose that the observed upregulation of both glutamate transporters may be a consequence of NF-κB pathway activation that seems to actively intervene in the protective compensatory action elicited by GA. On the other hand, NF-κB exerts a double face activity playing a detrimental role in inflammation. In effect, the present data showed a significant release of the proinflammatory cytokine TNF-α from GA-injured astrocytes into the extracellular environment that ultimately may affect neuronal survival. TNF-α release may serve as the catalyst for upstream harmful events, ultimately culminating in neurodegeneration disrupting normal cellular processes and promoting oxidative stress and disruption of Ca^{2+} homeostasis. Consequently, targeting TNF-α release or its downstream effectors could also provide potential therapeutic strategies for mitigating neuronal loss and slowing disease progression.

Recently, the implication of autophagy in astrocytes' function during AD has started to be investigated. Of note, the administration to transgenic mice of rapamycin, a drug inducing autophagy, reduces tau tangle pathology and GFAP level -measured as immunochemical signal- as a sign of reactive astrocyte limitation [87]. Complementary, expression of exogenous transcription factor EB (TFEB) in hippocampal astrocytes enhances Aβ degradation and reduces amyloid plaque deposition in the brain of mice expressing mutant human amyloid precursor protein (APP) [88].

Therefore, whether it is a neuron-mediated effect or astrocyte-directed, boosting autophagy appears to be a good pharmacological strategy useful to overcome the deposition of toxic aggregates in the brains of Alzheimer's patients. The present results showed that, entering the cell, glutamate may counterbalance the impairment of astrocytic autophagy induced by GA thus identifying a new non-cell-autonomous mechanism to control in AD therapy.

In conclusion, this study demonstrated that energetic deficiency induced by GA in cortical astrocytes may trigger a cascade of harmful events leading to the shift to reactive astrocytes. This astrocytic activation may compromise their physiological neuro-supportive role, adversely affecting neighboring neurons and contributing to the progression of neurodegenerative processes. Our findings highlight the potential of a neuroprotective strategies aimed at boosting astrocytic bioenergetics to counteract the hypometabolic state often observed in the prodromal phases of AD. In fact, due to the complexity of AD pathophysiology, it is crucial to study the AD pathomechanisms not only within contexts traditionally viewed from a neuron-centric perspective. A deeper understanding of the mechanisms downstream of metabolic defects in astrocytes could lead to innovative therapeutic approaches to slow or stop AD progression, greatly improving the lives of millions of patients worldwide.

4. Materials and methods

4.1. Drugs and chemicals

Media and supplements for cell cultures were obtained from Sial (Sial, Rome, Italy). 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazoliumbromide (MTT), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes), Glucose, 2-Amino-2-(hydroxymethyl)-1,3-propanediol (Trizma base), Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), β-Mercaptoethanol, glutamate (Glut), Poly-D-Lysine Hydrobromide (PDL) and Phosphate Buffer Saline (PBS), 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH), Bafilomycin A1 (Bafilo) were purchased from Sigma-Aldrich (Merck Millipore, Milan, Italy). Glyceraldehyde (GA) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit reaction solution was purchased from Roche (Basilea, Switzerland). Luminescence ATP Detection Assay System was purchased from Revvity (Waltham, MA, USA). MitoTracker CM-H2XRos and Fluo-4-AM dye were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Rhod-2-AM was obtained from Abcam (Cambridge, UK). (3*S*)-3-[[3-[[4-(Trifluoromethyl) benzoyl]amino]phenyl]methoxy]-L-aspartic acid (TFB-TBOA) was purchased from Tocris (Bristol, UK).

4.2. Primary cell culture and treatment

Primary rat cortical astrocytes (from this point onward, cortical astrocytes) were isolated from Wistar (Cat. 003WISTAR, Charles River, Lecco, Italy) rat pups (P2–P4) as previously described [19,21,89]. Ethical guidelines were strictly followed, ensuring compliance with the Ethics Committee for Animal Experiments, and adhering to the Italian Ministry of Health's regulations. Briefly, cortices were isolated from rat brains and placed in ice-cold PBS. The PBS was replaced with trypsin-EDTA 0.25% and subsequently the cortices were subjected to mechanical dissection by scissor cut, followed by enzymatic digestion obtained by incubating them for 15 min in a humidified incubator at 37°C with atmosphere at 5% CO_2. After trypsinization, the cells were centrifuged at 2000 rpm for 5 min. To neutralize the trypsin, the cortices were washed with media Dulbecco's Modified Eagle Medium (DMEM) 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. Cortical astrocytes were cultivated in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were cultured at 37 °C in an atmosphere with 5% CO_2. Cortical astrocytes were subjected to glyceraldehyde (GA, 1 mM) for 48 h, glutamate (Glut, 1 mM) was added after 24 h of GA exposure, while TFB-TBOA (TFB, 1 μM) was incubated 1 h before glutamate exposure and maintained until the end of the experimental protocol.

4.3. Cell viability

Cell viability was evaluated by LDH release and as mitochondrial activity measured by the MTT assay [19,70]. MTT assay was measured by detecting the absorbance value at a wavelength of 540 nm using a Victor Multilabel Counter plate reader from Perkin Elmer (Waltham, MA, USA). The outcomes were presented as percentages relative to the control value. LDH release was assessed by using the Cytotoxicity Detection Kit reaction solution at a specific wavelength of 490 nm using a Victor Multilabel Counter plate reader.

4.4. Quantification of ATP levels

Intracellular ATP levels were assessed by using a commercially available luciferase-luciferin kit [36]. 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 using a luminometer (Victor Multilabel Counter, Perkin Elmer), standardized to their corresponding protein content and presented as percentages relative to the control value.

4.5. Measurement of mitochondrial ROS production

Mitochondrial ROS production was observed using the MitoTracker

CM-H2XRos dye [21]. Cells were loaded with 300 nM dye concentration for 30 min 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. Images were acquired every 5 s, allowing for the tracking of basal ROS levels for 200 s. After the acquisition of the images, fluorescence intensity analysis was performed offline. The fluorescence values were expressed as percentages relative to the control value.

4.6. Immunofluorescence

Cells were exposed to MitoTracker Red CMXRos (Invitrogen) at a concentration of 300 nM for 30 min at a temperature of 37°C. Next, they were fixed using 3.7% formaldehyde for another 30 min at RT. After fixation, cells were permeabilized with Triton X-100 0.1% for a period of 5 min at RT. Then, cells were subjected to a 1.5 h incubation at RT with primary antibodies. To identify the immunoreactions, a conjugated secondary antibody (Alexa Anti-Mouse 488, Thermo Scientific) with a 1:200 dilution, was employed. Images were acquired with a 510 LSM microscope (Carl Zeiss) equipped with a META detection system and an inverted microscope Eclipse Ti2-E supplied with an AX confocal system (Nikon, Japan) at 60x magnification.

4.7. Enzyme-linked immunosorbent assay (ELISA)

The evaluation of TNFα release was conducted using the Rat TNF alpha ELISA Kit (Invitrogen). 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.

4.8. 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 quantified 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 min. Proteins were then separated via electrophoresis on an 8–15% SDS-polyacrylamide gel and then blotted onto a nitrocellulose membrane (Bio-Rad). Following the blocking phase (1 h at RT with 5% non-fat dry milk in PBS buffer), the membranes were placed at 4°C for an overnight period with the appropriate primary antibody (Table 1). To detect the immunoreactions, the membranes were incubated with the suitable secondary antibody, which was conjugated to horseradish peroxidase (Goat anti-rabbit IgG-HRP, sc-2357, Santa Cruz; Goat anti-Mouse IgG (H $^+$ L) HRP Cat. 62–6520, Invitrogen), for 1 h 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). Band densities were analyzed with Uvitec Nine Alliance analysis software.

4.9. Quantification of basal intracellular and mitochondrial Ca^{2+} levels

Intracellular and mitochondrial basal Ca^{2+} levels were monitored by single-cell computer-assisted videoimaging using a LSM 510 confocal system (Carl Zeiss) [21]. In brief, cortical astrocytes were loaded with 1 μM Fluo-4-AM or 2 μM Rhod-2-AM in the medium for 45 min at 37 °C. After loading, cells were washed in standard buffer solution for an additional 10 min, placed into a perfusion chamber mounted onto the stage of an inverted Zeiss Axiovert 200 microscope and perfused with standard buffer solution (in mM: 140 NaCl, 5 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 10 HEPES, 5.5 glucose, buffered to pH 7.4 with NaOH) for about 200 s at 37 °C using a heated microscope stage and climate box from PeCon GmbH. Peristaltic pump was used to deliver solutions; images were acquired every 5 s and then analyzed offline. Fluo-4-AM was excited at 488 nm, and the emitted fluorescence was recorded at 505–530 nm, while Rhod-2- AM was excited at 543 nm, and fluorescence emission was measured from 560 to 600 nm.

Free $[Ca^{2+}]$ was calculated by using Grynkiewicz equation [90]: $[Ca^{2+}] = Kd\ (F-F_{min})\ /\ (F_{max}-F)$, where Kd of Fluo-4-AM and Rhod-2-AM are 345 nM and 570 nM respectively. $[Ca^{2+}]$ was reported as percentages relative to the control value [13].

4.10. Statistical analysis

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

Table 1
List of primary antibodies used in this study.

Antibody	Supplier	Catalog number	Species	Type	Application	Dilution
Anti p65	Cell Signaling Technology Inc. (Danvers, MA, USA)	3033 (RRID:AB_331284)	Rabbit	Monoclonal	WB	1:1000
Anti-β-Actin	Sigma-Aldrich (Milan, Italy)	A5316 (RRID:AB_476743)	Mouse	Monoclonal	WB	1:10000
Anti GFAP	Antibodies (Stockholm, Sweden)	A85419 (RRID: AB_2752917)	Rabbit	Polyclonal	IF	1:100
Anti Glutamine Synthetase (GS)	Invitrogen (ThermoFisher Scientific Inc., Waltham, MA, USA)	PA1–46165 (RRID: AB_2263362)	Rabbit	Polyclonal	IF	1:500
Anti S100 β	Antibodies (Stockholm, Sweden)	A253116 (RRID not available)	Mouse	Monoclonal	IF	1:500
Anti GLAST	Immunological Sciences (Rome, Italy)	AB-82224 (RRID not available)	Rabbit	Polyclonal	WB / IF	1:1000 / 1:100
Anti GLT1	Immunological Sciences (Rome, Italy)	AB-82459 (RRID not available)	Rabbit	Polyclonal	WB / IF	1:1000 / 1:100
Anti RAGE	Antibodies (Stockholm, Sweden	A11444 (RRID not available)	Rabbit	Polyclonal	WB	1:1000
Anti AGE	Abcam (Cambridge, UK)	AB23722 (RRID:AB_447638)	Rabbit	Polyclonal	WB	1:1000
anti-pAMPK α	Cell Signaling Technology Inc. (Danvers, MA, USA)	2531 (RRID:AB_330330)	Rabbit	Polyclonal	WB	1:1000
anti-p62/SQSTM1	Novus Biologicals (Littleton, CO, USA)	NBP1–48320 (RRID: AB_10011069)	Rabbit	Polyclonal	WB	1:1000
anti-LC3B	GeneTex Inc. (Irvine, CA, USA)	GTX127375 (RRID: AB_11176277)	Rabbit	Polyclonal	WB	1:1000
pTau	Invitrogen (ThermoFisher Scientific Inc., Waltham, MA, USA)	MN1060 (RRID:AB_223652)	Mouse	Monoclonal	IF	1:1000

Author contributions

SP, AS and SM conceived and planned the experiments. SP, AP, TZ, VT, AS, SM, VL, PC contributed to data acquisition and analysis. SP, AP, AS and SM wrote the manuscript. SP, AP, TZ, VT, SM and VL contributed to the interpretation of the results. SP, VL and SM acquired funding. All authors reviewed and edited the manuscript and approved the final version of the manuscript submitted for publication.

Funding

This study was supported by “Ministero dell’Istruzione, dell’Università e della Ricerca” (MIUR) Grant 2022ZTKFXS to VL; Ricerca Finalizzata/Sanità - Regione Sardegna “Modulation of neuro-inflammation and astrocyte activity in Alzheimer's disease: functional consequences and therapeutic perspectives” to SM; “Ricerca Scientifica di Ateneo” (RSA) Grant (2022–2023) to SM, VL and SP.

CRediT authorship contribution statement

Alessandra Preziuso: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Silvia Piccirillo:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Pasqualina Castaldo:** Validation, Formal analysis. **Valentina Terenzi:** Validation, Formal analysis. **Tiziano Serfilippi:** Validation, Formal analysis. **Simona Magi:** Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Agnese Secondo:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Vincenzo Lariccia:** Validation, Funding acquisition, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors were grateful to Tatiana Spadoni, Center of Confocal and Electronic Microscopy and CLEM, University "Politecnica delle Marche", Ancona, Italy, for her help in the immunocytochemistry images’ acquisition.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2026.119473.

Data availability

Data will be made available on request.

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