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Effects of Choline Alphoscerate on neurotoxicity in a model of Alzheimer's disease

Ph.D. Thesis

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List of abbreviations

AD	Alzheimer’s disease
Aβ	Amyloid-beta
APP	Amyloid precursor protein
BBB	Blood brain barrier
CNS	Central nervous system
MCI	Mild cognitive impairment
NF-κB	Nuclear transcription factor-κB
NFTs	Neurofibrillary tangles
TNF	Tumor Necrosis Factor
p-Tau	Phosphorylated Tau

Abstract

Alzheimer's disease (AD) is the most common cause of dementia worldwide, characterized by extreme unmet needs and a huge disease burden. Cerebral atrophy, amyloid plaques and neurofibrillary tangles represent the main pathological characteristics of the AD brain.

Despite remarkable research efforts to unravel pathophysiological mechanisms of AD, a disease-modifying therapeutic option is still today far from clinical practice, as numerous phase III clinical trials targeting amyloid-beta (Aβ) and other disease markers have failed to improve clinical outcomes of AD patients enrolled. The identification of reliable biomarkers that reflect disease progression is crucial to design disease-modifying therapies able to act at its earliest preclinical stage for better management of AD patients. Elevated levels of inflammatory markers in patients with AD and the identification of AD risk genes associated with innate immune functions suggest that inflammation holds a crucial role in AD pathogenesis, implying that immune pathways could represent therapeutic targets.

Although inflammation is intended to be protective, an excessive inflammatory response can cause or contribute to tissue damage: neuroinflammation is to be considered as an inflammatory response in the CNS secondary to a neuronal insult and, in AD especially in the early stages, is a vicious cycle of glial trigger, release of proinflammatory factors and neuronal damage.

The approach proposed in this work has been to focuses our interest on the molecule of α-GPC (alpha-glyceryl-phosphorylcholine), significantly effective in enhancing Ach synthesis and release, probably due to its ability to cross the blood brain barrier (BBB) and contribute to nerve cell/synaptic membrane reorganization.

Considering the potential protective action of α-GPC on cholinergic neurotransmission, the first purpose of our study has been to evaluate both its effects on Aβ-induced neurotoxicity in an *in vitro* model and the potential neuroprotective capabilities of the molecule in an *in vivo* model of AD (3xTg-AD).

General introduction

1. Alzheimer’s Disease: a general background

Dementia has become a global challenge for public health.

Alzheimer's disease (AD) is the most common type of dementia, accounting for at least two-thirds of cases of dementia in people age 65 and older. Alzheimer's disease is a neurodegenerative disease with insidious onset and progressive impairment of behavioral and cognitive functions including memory, comprehension, language, attention, reasoning, and judgment. It is the sixth leading cause of death in the United States. There is no cure for Alzheimer's disease, although there are treatments available that may improve some symptoms [1].

Symptoms of Alzheimer's disease depend on the stage of the disease. The initial and most common presenting symptom is episodic short-term memory loss with relative sparing of long-term memory and can be elicited in most patients even when not the presenting symptom. Short-term memory impairment is followed by impairment in problem-solving, judgment, executive functioning, lack of motivation and disorganization, leading to problems with multitasking and abstract thinking [2,3]. In the early stages, impairment in executive functioning ranges from subtle to significant. This is followed by language disorder and impairment of visuospatial skills. Neuropsychiatric symptoms like apathy, social withdrawal, disinhibition, agitation, psychosis, and wandering are also common in the mid to late stages. Difficulty performing learned motor tasks (dyspraxia), olfactory dysfunction, sleep disturbances, extrapyramidal motor signs like dystonia, akathisia, and parkinsonian symptoms occur late in the disease. This is followed by primitive reflexes, incontinence, and total dependence on caregivers [1,5].

The neuropathology of AD manifests in several features.

Although the brain of AD patients does not show any macroscopic alteration that can be considered diagnostic [6], a typical symmetric pattern of cortical thinning and atrophy, predominantly affecting the medial temporal lobes, and subsequent enlargement of the frontal and temporal horns of the lateral

ventricles (*ex vacuo hydrocephalus*) [7] are considered highly suggestive of AD (**Figure 1**).

The cardinal microscopic pathological hallmarks of the disease, currently used for diagnostic interpretation, are represented by amyloid plaques that contain extracellularly deposited amyloid β (Aβ) obtained from cleaved amyloid precursor protein (APP), and neurofibrillary tangles (NFTs) generated by intracellular accumulation of hyperphosphorylated and misfolded tau protein. These neuropathological features showa different degree of correlation with the dementia severity or duration in AD [8].

Aβ plaques are detectable in the brain many years or even decades before dementia onset [9] but appear to have only subtle effects on cognition and brain health in humans [10,11]. In contrast, the spreading of tau neurofibrillary tangles, a process that animal models have suggested may be accelerated by the presence of brain Aβ plaques, is strongly correlated with local neurodegeneration and frank cognitive impairment [12].

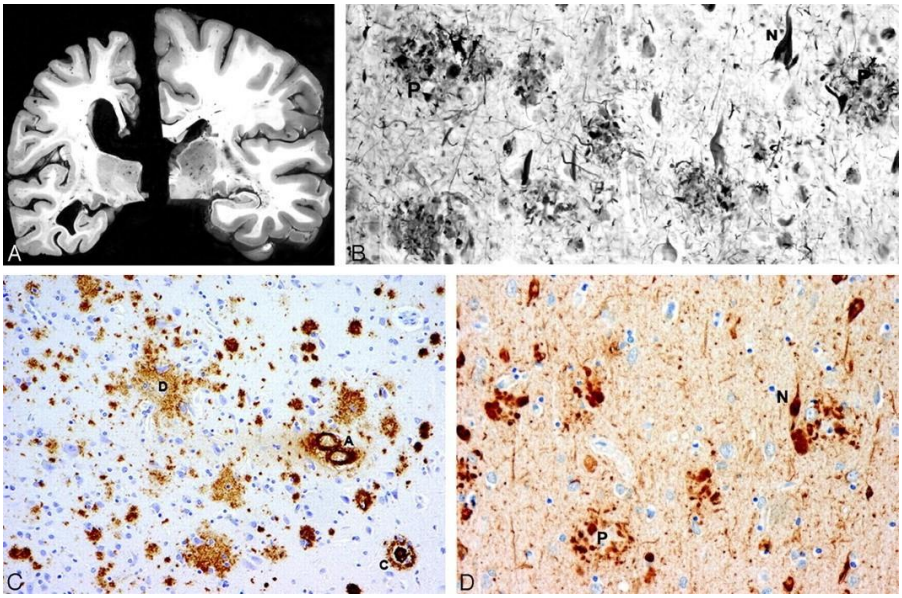

Figure 1. Macroscopic and microscopic features of Alzheimer's brain. A. Brain atrophy. Sectionof the cerebral hemisphere of a 70-year-old AD patient and, on the right, a healthy aged brain. The AD brain shows marked atrophy, often accompanied by enlargement of the frontal and temporal horns of the lateral ventricles, and a small hippocampus. **B**. Neurofibrillary tangles (N) and A□ plaques (P) in the hippocampus. Image obtained by silver impregnation. **C**. β-amyloidosis in the frontal lobe: a diffuse plaque (D), a cored plaque (C), and cerebral amyloid angiopathy (A). **D**. Neurofibrillary tangles (N) and A□ plaques (P) in the frontal lobe. [13]

Despite the efforts made to validate approaches for early diagnosis, the advent of sophisticated neuroimaging techniques [14], and the search for reliable biomarkers [15,16], to date, clinical AD dementia cannot be definitively diagnosed until post-mortem neuropathological examination [4].

Although remarkable research efforts have been made to uncover the pathophysiological mechanisms of AD and to further translate these findings into the clinic, currently available Food and Drug Administration (FDA)-approved pharmacotherapies for AD (listed in **Table 1**) [17] represents only symptomatic treatments with no disease-modifying potential, licensed for the management of cognitive impairment and for the dementia stage of AD [3].

Except for memantine, which blocks excessive stimulation of N-methyl-D-aspartate (NMDA) receptors in the brain preventing nerve cells damage, these drugs include cholinesterase inhibitors actively involved in counteracting the neurotransmitter imbalances typical of the disease [18].

Drug	Time (approved by FDA)	Chemical class	Action	Type of inhibition	Route of administration	Indication	Status
Tacrine	1995	Alkaline	AChE inhibitor	Rapidly reversible	Oral or rectal	_	Withdrawal
Donepezil	1996	Piperidine	AChE inhibitor	Rapidly reversible	Oral	Mild- moderately (mod) AD	Approved
Rivastigmine	1997	Carbamate	AChE and BChE inhibitor	Pseudoreversible	Oral or transdermal patch	Mild-mod AD	Approved
Galantamine	2001	Phenanthrene alkaloid	AChE inhibitor	Rapidly reversible	Oral	Mild-mod AD	Approved
Memantine	2003	Glutamatergic modulator	NMDA antagonist	N/A	Oral	Mod-severe AD	Approved

Table 1. FDA-approved pharmacotherapies for Alzheimer's disease.

Available data from a wide number of clinical trials in which various hypotheses for AD have been tested [2,19], and the limited progress of therapeutics with potentially disease-modifying properties in phase III clinical trials (**Figure 2**), suggest that it is time to adopt alternative strategies for AD treatment [20].

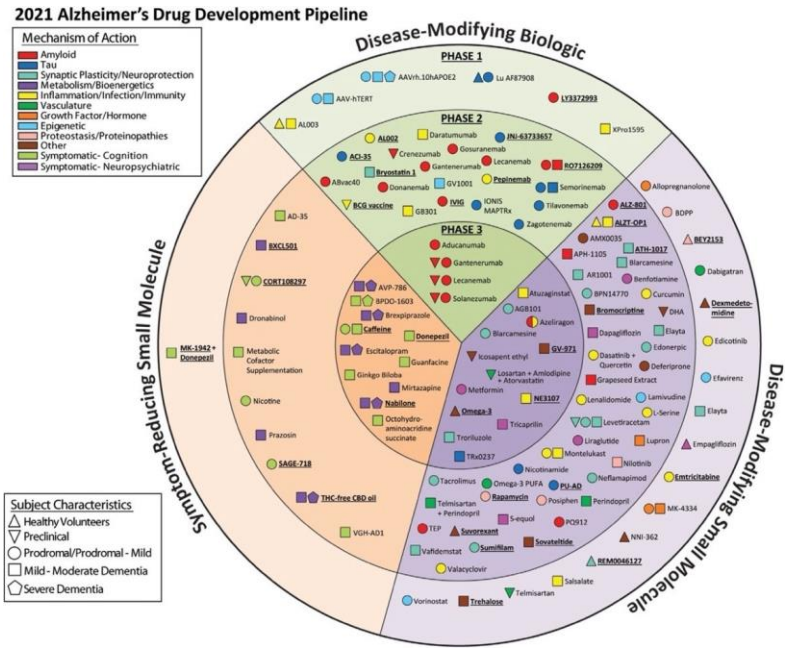

Figure 2. Agents in clinical trials for treatment of Alzheimer's disease in 2021. Phase 1, 2 and 3 agents are respectively shown in the outer, middle, and inner rings. Agents in green area are biologics; agents in purple are disease-modifying small molecules; agents in orange are symptomatic agents addressing cognitive, behavioral and neuropsychiatric symptoms; the shape of the icon showsthe population of the trial; the icon color shows the Common Alzheimer's Disease Research Ontology (CADRO)-based class of the agent [19].

Most of the failed phase III trials intervened on patients with mild-to-moderate symptomatic AD, a stage in which significant and irreversible synaptic and neuronal loss has already occurred, and the pathological cascade would likely be very difficult to reverse [17,21].

In this scenario, the identification of reliable biomarkers that reflects disease progression is crucial to design disease-modifying clinical interventions able to act early in the disease continuum, either during the preclinical or mild cognitive impairment (MCI) phases [1]. At these stages, therapies might have a better chance of changing disease trajectory [17,22,23].

In short, at present, all the new strategies in AD drug development seems to shift the focus from treatment to prevention by examining the potential neuroprotective activity of disease-modifying drugs in the pre-symptomatic stages of AD, with the help of biomarkers that predict disease progression before the development of overt dementia.

2. Neuroimmune interactions in Alzheimer's Disease

Over the years, several hypotheses have been proposed to unveil the complex pathological mechanisms underlying AD-related neurodegeneration [2,24]. However, the ultimate etiology of AD remains obscure.

AD is pathologically characterized by senile plaques and intracellular neurofibrillary tangles (NFTs), consisting of β-amyloid (Aβ) aggregates and hyperphosphorylated microtubule-associated protein Tau, resulting in neuronal dystrophy and loss, respectively.

Neuroinflammation, defined as activation of glial cells, such as microglia and astrocytes, and subsequent production of inflammatory factors such as cytokines and chemokines surrounding senile plaques and affected neurons in the brains, is observed in AD patients.

Genome-wide association studies (GWAS) of AD risk genetic variants revealed that a large proportion of identified genes were closely related to immune responses, and that their expressions were enriched in microglia and macrophages.

Microglia, one of the resident innate immune cells in the central nervous system (CNS), originate from erythromyeloid progenitor cells in the embryonic yolk sac.

Microglia plays an important role in immune surveillance, by phagocytotic clearance of pathogens, dead cells, cellular debris, and protein aggregates like those of Aβ, and help maintain homeostasis in the CNS. Microglia also contributes to brain development and its maintenance by participating in synaptic pruning and myelination. Meanwhile, once microglia respond to their stimuli, their gene expression profiles undergo distinct alterations, with an immediate production of various inflammatory cytokines and mediators and a change in their morphology to an amoeboid shape. It is suggested that long-lasting neuroinflammation causes a decline in homeostatic functions of microglia, resulting in neuronal loss and neurodegenerative diseases. However, it is unknown whether the loss of homeostatic functions of microglia can be correlated with the degree of neurodegeneration and neuronal loss.

These observations and the discovery of elevated levels of inflammatory markers associated with cognitive decline in AD patients [25,26] suggest the

existence of tight interactions of immunological mechanisms within the central nervous system (CNS) [27].

As it is now well-recognized that AD is a disorder not completely restricted to pathology and biomarkers within the brain, it might become necessary for AD to be studied as a generalized systemic disorder by targeting surrogate tissues [28].

After years of failed therapeutic attempts targeting Aβ and other disease markers, several studies suggest that neuroinflammation holds a crucial role in AD pathogenesis, implying that immune pathways could represent primary therapeutic targets [26].

2.1. Focus on neuroinflammation and mediators of immune/inflammatory response in Alzheimer's Disease

Despite the established perception of the brain as a site of immune privilege had hindered research and therapeutic approaches that involve immune pathways for years [29], only recently neuroinflammation and the immune response in the CNS have been appreciated as major driving forces in AD pathogenesis [30,31].

The immune privilege is not absolute, and it is now accepted that cells of the CNS are sensitive to the inflammatory events occurring both within the brain and in the periphery, as well as to the infiltration of peripheral immune cells [32,33].

The inflammatory response in AD is a double-edged sword. Primarily, it constitutes a self-defense reaction aimed at eliminating harmful stimuli and restoring tissue integrity and becomes detrimental when a chronic response is mounted [34,35]. In particular, brain damage due to Aβ and NFTs in AD triggers a local immune response mediated by astrocytes and microglia that respond to these stimuli producing various mediators of the inflammatory/immune response (i.e., pro-inflammatory cytokines, acute-phase proteins, and complement components) [36,37].

When such a first immune-related process aimed at removing the harmful stimuli is not completely resolutive and the inflammatory stimuli persist, glia-mediated mechanisms remain trapped in a vicious cycle characterized by chronicized release of pro- inflammatory cytokines and chemokines [38,39].

These inflammatory mediators and all the pathological components of the unresolved response promote the recruitment of the peripheral leukocytes, which infiltrate the brain via the compromised blood-brain barrier (BBB). Immune cells infiltrating the brain parenchyma release neurotoxic and proinflammatory factors that act on glial cells, fueling any existing central inflammation [39].

The neuroinflammatory process increases disease severity by exacerbating Aβ and tau pathologies [34,36].

Moreover, the scenario of an inflammatory response not limited only to the CNS but also involving the peripheral compartment suggests that AD could be considered as a generalized systemic disorder [30].

Although the description of pathways for periphery-brain communication is beyond the scope of the present work and have been reviewed in detail elsewhere [40,41], inflammatory reaction in peripheral tissues can induce the production of cytokines which in turn communicate with the brain via blood flow causing damage of BBB integrity. Subsequent cerebral infiltration of peripheral immune cells further contributes to brain pathology and synthesis of inflammatory mediators in the brain parenchyma [2].

In this scenario, the study of pathological changes occurring beyond the brain could offer new opportunities for the early diagnosis of AD and lead to the design of specific therapeutic strategies.

3. Cholinergic precursors

Changes in cholinergic function are implicated in the pathogenesis of learning and memory alterations occurring in adult-onset cholinergic dysfunction including dementia disorders. Brain cholinergic pathways are not the only neurotransmitter system affected in cognitive dysfunction common of Alzheimer's disease or vascular dementia, but their involvement in cognition is commonly accepted.

Studies of the brain of patients suffering from Alzheimer's disease have shown marked loss of the acetylcholine synthesizing enzyme choline acetyltransferase and of nicotinic cholinergic receptors. A correlation between the loss of cortical cholinergic synapses and between this loss and the decrease of high affinity cholinergic receptors was reported. These findings have contributed to the development of the so-called cholinergic hypothesis of geriatric memory disfunction.

They served also as the conceptual basis to consider restoration of deficient cholinergic neurotransmission involving primarily the basal forebrain as a possible treatment of adult-onset dementia disorders.

Cholinergic precursors have represented an old approach to treat cholinergic dysfunction and cognitive decline in adult-onset dementia.

Many of these precursors were early leaved because their efficacy was not clearly demonstrated.

This is not true for some cholinergic precursors including choline alphoscerate, a cholinergic precursor available in the pharmaceutical market of several countries, which has been studied both in preclinical paradigms and in clinical trials [42].

3.1. Choline alphoscerate: an old choline with a still interesting profile as cognition enhancing agent

Choline alphoscerate or alpha-glyceryl-phosphorylcholine (ATC code N07AX02) (α-GPC) is a semisynthetic derivative of lecithin. Following oral administration, it is converted to phosphorylcholine, a metabolically active form of choline able to reach cholinergic nerve terminals where it increases acetylcholine synthesis, levels and release.

Although choline alphoscerate is in the pharmaceutical market since 1987, the interest on it was apparently reduced after the introduction in therapy of

cholinesterase inhibitors. In the last 10 years a renewed attention on the compound was seen with preclinical studies, clinical investigations and review articles published in literature [42].

Choline alphoscerate interferes with brain phospholipid metabolism and increases brain choline and acetylcholine levels and release.

Preclinical studies have demonstrated that choline alphoscerate increases the release of acetylcholine in rat hippocampus, facilitates learning and memory in experimental animals, improves brain transduction mechanisms and decreases the age-dependent structural changes occurring in the rat frontal cortex and hippocampus.

Moreover, the compound demonstrated to improve cognitive deficits and to reverse mnemonic disorders; based on the above evidence, the central parasympathomimetic activity of the molecule was defined, suggesting its possible clinical use in patients affected by cognitive decline.

Chapter 1

EFFECTS OF MICROGLIAL PHENOTYPIC SWITCHING IN ALZHEIMER'S DISEASE: POTENTIAL IMPLICATION OF CHOLINE ALPHOSCERATE

Effects of microglial phenotypic switching in Alzheimer's disease: potential implication of choline alphoscerate.

MS submitted to Cells

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia, characterized by progressive memory loss and cognitive impairment. Despite the increasing number of affected patients, treatment options remain poor. The cholinergic system plays a crucial role in physiological condition such as memory, attention and learning; on the other hand, an altered cholinergic transmission is a very important factor in many neurodegenerative disorders, including AD. We have previously shown that Choline alphoscerate (α-GPC) protects neuronal cell line SH-SY5Y from death challenged with Aβ. Here, we investigate the effects of α-GPC as an efficient modulator of α7 nAChR upon microglial phenotype in response to Aβ. The BV2 murine microglial cell line was used as an in vitro model of AD. BV2 cells were pre-treated for 1h with α-GPC (1 μM) and treated for 24, 48, and 72h with $A\beta_{1-42}$ (5 μM) /α-BTX (100 nM). Fluorescent immunocytochemistry and Western blot analysis showed that α-GPC was able to re-balance $A\beta_{1-42}$ mediated inflammation and increased the expression of anti-inflammatory molecules. In addition, α-GPC, exerted its anti-inflammatory role directly activating α7 nAChR receptors, suggested by the increased $[Ca^{2+}]_{i.}$ current. Results of this study show that α-GPC treatment is associated with substantial restraint of the Aβ-mediated inflammatory effects. Thus, modulation of the cholinergic transmission could be envisioned as a pharmaco-therapeutic target for improving outcomes in inflammatory neurodegenerative disorder, such as Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) is the most common of the age-related neurodegenerative disorders known to cause impairments in cognitive processes, particularly memory and attention. Critical brain regions for the maintenance and modulation of such brain functions are cerebral cortex and hippocampus, whose cholinergic innervation is mainly provided by the cholinergic nuclei of the basal forebrain (BFCN). The latter are highly susceptible to Alzheimer's disease, and their selective degeneration, resulting in a dysfunction of cholinergic neurotransmission, led to the formulation of the cholinergic hypothesis of age-related cognitive dysfunction (**Martinez JL 2021**) (**Hampel H, 2018**). Recognition of the critical role of altered cholinergic transmission in the pathophysiology of the disease has led to the development of therapeutic strategies aimed at restoring cholinergic function, offering a glimmer of hope for improving the quality of life of people affected by this debilitating condition. Cholinergic precursors represent the first potential approach to counteracting the cholinergic impairment and cognitive loss seen in various forms of dementia (**Traini E, 2013**). Among them, choline alphoscerate (L-alpha-glycerylphosphoxycholine; α-GPC) given its high choline content (41% choline by weight) and ability to cross the blood-brain barrier, is one of the most widely used sources of choline (**Kansakar U, 2023**). Indeed, it has been found to be effective in improving the synthesis and release of acetylcholine (**Traini E, 2013**). However, although previous studies have shown that the *in vitro* administration of α-GPC is able to protect neurons from the toxicity induced by Aβ (**Burgaletto 2021**), only few studies are focused on the characterization of the mechanism behind this effect. α-GPC's neuroprotective effect against Aβ-mediated neurotoxicity likely involves enhanced anabolic processes responsible for the synthesis of membrane phospholipids, which improve synaptic membranes fluidity, and its role as a precursor of acetylcholine, thereby increasing its availability.

Activation of nicotinic acetylcholine receptors (nAChRs) by acetylcholine in the brain frontal cortex has been shown to be essential for functions such as attention and working memory (**Hurst 2013**); **Galvin VC, 2020**). Particularly, the nicotinic alpha-7 acetylcholine receptor (α7 nAChR) has been demonstrated to play an important role in AD **(Ma KG, 2019).** Specifically, α7 nAChR is a ligand-activated

ion channel expressed in different brain regions, including cerebral cortex and hippocampus, responsible for cognitive functions **(Ma KG, 2019).** α7 nAChR regulates neural circuit plasticity, neuronal differentiation, proliferation, and apoptosis. In addition, α7 nAChR also has vital functions in the glia cells (**Orr-Urtreger A, 2000)**. In fact, such receptor is not only expressed on neurons, but also on mature dendritic cells and microglia cells (**Clarke PB, 1985)**. Microglial cells exert an immune-surveillance function essential in the CNS, acting as "sentinels", promptly detecting signs of tissue damage or pathogen presence, setting into motion an inflammatory response to fight against deviation from homeostasis (**Yin J, 2017**); (**Burgaletto C, 2020)**. Although microglia activation is often neuroprotective, persistent stimulation of these cells can trap them in a vicious cycle, marked by chronic release of pro-inflammatory cytokines that trigger a cascade of toxic events, ultimately leading to neurotoxicity (**Burgaletto C, 2020**); (**Cherry JD, 2014**); (**Hanisch U-K, 2007**); (**Perry VH, 2010**). Due to activation, microglia can display a broad spectrum of phenotypes ranging from the classical pro-inflammatory and potentially cytotoxic phenotype M1 to the alternative anti-inflammatory and neuroprotective phenotype M2. Thus, microglia with their pivotal involvement in neuroinflammatory processes, play a key role in the interplay between inflammation and neurodegeneration. Notably, α7 nAChR has been increasingly recognized as a crucial mediator in the above-mentioned context (**Akiyama H, 2000**). Indeed, several studies suggest that α7 nAChR activation inhibits NF-κB-dependent pro-inflammatory cytokines production and release (**St-Pierre S, 2016**); (**Ma Z, 2019**); (**Young KF, 2009**). Given this background, the aim of this study is to assess the role of α-GPC treatment on microglial phenotypic switching. Specifically, the *in vitro* effects of α-GPC (used as an indirect agonist of α7 nAchR) on Aβ-induced microglial switching in BV2 cells, a murine microglial cell line, was investigated. In addition, the different test groups were treated with α-bungarotoxin (a selective α7 nAchR antagonist) to verify the receptor's direct implication in mediating this mechanism. The M1 and M2 microglial activation phenotypes were studied qualitatively through immunofluorescence techniques, with the aim of evaluating the expression of specific markers, and quantitatively through Western blot analysis, with the ultimate objective of evaluating the cytokines production related to both phenotypes.

MATERIALS AND METHODS

Drugs and chemicals

Culture media were purchased from Thermo Fisher Scientific, Inc, Massachussets, USA.

Italfarmaco, Milano, Italy, provided α-GPC. Amyloid β protein lyophilized fragment 1–42 was obtained from Sigma-Aldrich, St. Louis, MO, USA. All other chemicals were of the highest commercial grade available.

Preparation of $A\beta_{1-42}$ Oligomers

$A\beta_{1–42}$ oligomers were generated as the previously described method (**Fa M, 2010**). Briefly, under the fume hood, the $A\beta_{1–42}$ lyophilized peptide was initially dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma-Aldrich) to a final concentration of 1 mM, aliquoted, and incubated at room temperature (RT) for 2 h to allow monomerization. Traces of HFIP were removed under vacuum by a SpeedVac centrifuge (800× *g*, RT), and the thin clear peptide film was stored over desiccant at −80 °C. For oligomerization, the aliquoted peptide film was dissolved in dimethyl sulfoxide (DMSO) to 5 mM. The peptide in DMSO was diluted directly into sterile phosphate buffered saline (PBS, 1X) at 100 μM and incubated at 4 °C for 12 h to make the oligomeric form of $A\beta_{1–42}$. Following incubation, $A\beta_{1-42}$ samples were immediately used for the cell treatment or aliquoted and stored at –20 °C until their use.

Cell cultures

The murine BV2 microglia cell line was obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; High Glucose) supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 100 g/mL penicillin, 100 g/mL streptomycin, 1% (v/v) sodium pyruvate. Cultures were kept at 37°C in humidified 5% CO2 /95% O2 atmosphere.

Cell Viability Test

Cell viability was determined by using 3-[4,5 dimethylthiazol-2-yl]-2,5-

diphenyltetrazolium bromide (MTT) assay. A total of $5x10^3$ cells per well were plated on 96-well plates. Culture medium was changed to a medium containing 0,5 mg/mL MTT (Sigma-Aldrich, Milan, Italy), and cell viability was measured by the reduction of MTT solution. After 3h of incubation at 37 °C, the solution was removed and dimethylsulfoxide (DMSO) was added to obtain cell lysis and to dissolve blue formazan crystals resulting from MTT reduction by viable cells' mitochondrial activity. The optical density of the supernatants was measured at 545 nm with a VarioskanTM Flash Multimode Reader. Data were expressed as the mean percentage of viable cells versus control. Experiments were performed in triplicate at least twice.

Western blot analysis

For protein extraction and Western blot (WB) analysis, cells were lysed in buffer containing 150mM NaCl, 50mM Tris-HCl (pH 7.5), 5mM EDTA, 1mM Na3VO4, 30mM sodium pyrophosphate, 50mM NaF, 1mM acid phenyl-methyl-sulphonyl-fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 10% glycerol, and 0.2% TritonTM X-100. The lysates were then centrifuged at 14000 rpm for 10 min at 4°C, and supernatants were collected. Protein concentration of the supernatant was determined by Bradford method (**Bradford MM, 1976**). Equal amounts of protein (30 μg) were separated by 8–12% SDS-PAGE gels and transferred onto Hybond ECL nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). Membranes were blocked for 1 h at RT with 5% non-fat dry milk plus 0.05% Tween 20 (PBS-T). For primary antibody reactions a rabbit anti-IL10 antibody (Abbiotec, San Diego, CA, USA, 250713; 1:250) or a rabbit anti-TNF-α antibody (Novus Biologicals, Littleton, Colorado NB600-587; 1:1000), or a rabbit anti-nAchR α7 (Abcam, Cambridge, UK, ab216485; 1:250) were added to membranes and stayed overnight at 4 °C on an orbital shaker. Then, the membranes were washed with PBS-T and were probed with the appropriate horseradish peroxidase-conjugated secondary antibody (Amersham Life Science, Buckinghamshire, UK) for 1 h at RT. β-actin (Santa Cruz Biotechnology Inc, Santa Cruz, Calif. sc-47778; 1:500) was used as control to validate the amount of protein loaded in the gels. After washing three times with PBS-T, protein bands were visualized by means of ECL chemiluminescence assay at dark (Amersham Life Science, Thermo Fisher

Scientific) and scanned with the iBright FL1500 Imaging System (Thermo Fisher Scientific). Densitometric analysis of band intensity was evalueted on immunoblots by using IMAGE J software (https://imagej.nih.gov/ij/).

Fluorescent immunocytochemistry

After treatment, BV2 cells were fixed for 15 min in 4% paraformaldehyde, permeabilized for 7 min with 0.1% Triton X-100, and then blocked for 30 min with 1% BSA. Cells were incubated for 1 hour at RT with mouse anti-CD86 (Santa Cruz, sc-28347, 1:250) or mouse anti-CD68 (Santa Cruz, sc-20060; 1:250) or a rabbit anti-IL-10 antibody (Abbiotec, 250713; 1:200) or a rabbit anti-TNF-α antibody (Novus Biologicals, NB600-587; 1:100) or a rabbit anti-nAchR α7 (Abcam, ab216485; 1:500). After washing in PBS three times for 5 minutes each, cells were incubated for 1 h at RT in the dark with the appropriate fluorescent-labelled secondary antibodies: Alexa Fluor 488 donkey anti-mouse (Thermo Fisher Scientific,) or Alexa Fluor 546 donkey anti-rabbit (Thermo Fisher Scientific,), or Alexa Fluor 488 donkey anti-rabbit (Thermo Fisher Scientific). Finally, for nuclear staining and stabilization of fluorescent signals, slides were covered in mounting medium (Fluoroshield with DAPI; Sigma-Aldrich, Milan, Italy) and secured with a coverslip. Fluorescence images were captured with a Zeiss Observer.Z1 microscope equipped with the Apotome.2 acquisition system (Zeiss LSM 700, Germany)

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ was measured by single-cell Fura-2 acetoxymethyl-ester (AM) videoimaging, as previously described (**Boscia F, 2009**). BV2 cells, placed on glass coverslips, were loaded with 10 μmol/L Fura-2AM for 30 minutes at 37 °C in normal Krebs solution containing 5.5 mMKCl, 160 mMNaCl, 1.2 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, and 10 mM HEPES-NaOH (pH 7.4). $[Ca^{2+}]_i$ was measured by a live-imaging system composed of the inverted Zeiss Axiovert 200 microscope (Carl Zeiss), MicroMax 512BFT cooled CCD camera (Princeton Instruments), LAMBDA10-2 filter wheeler (Sutter Instruments), and Meta-Morph/MetaFluor Imaging System software (Universal Imaging). After loading, samples were alternatively illuminated at 340 nm and 380 nm wavelengths. Drug effect on $[Ca^{2+}]_i$ was evaluated as D% peak increase over basal values in the

absence or presence of α-bungarotoxin. BV2 cells were treated with the toxin for 5 min before the registration and then analyzed.

Patch-clamp electrophysiology

Choline alphoscerate (α-GPC) activity on α7 nicotinic acetylcholine receptor was studied by the whole-cell patch clamp in BV2 cells. nAChR currents were recorded from BV2 cells by patch-clamp technique in whole-cell configuration using a commercially available amplifier Axopatch200B (Molecular Devices, CA, USA) and data were acquired with a Digidata1322A acquisition system (Molecular Devices, CA, USA) and pCLAMP 10 software (Molecular Devices, CA, USA) (REF). The peak current amplitude and charge movement (area-under-curve, AUC) induced by agonist application were measured using ClampFit 10 (Molecular Devices, CA, USA). Patch borosilicate glass pipettes were prepared with a puller (Narishige, PC-10, Tokyo, Japan). The resistance of the pipette was 4-5 MΩ. The dialyzing pipette solution contained the following (in mM): 100 Cs-gluconate, 10 TEA, 20 NaCl, 1 Mg-ATP, 0.1 $CaCl_2$, 2 $MgCl_2$, 0.75 EGTA, and 10 HEPES, adjusted to pH 7.2 with CsOH. The cells were perfused with external Ringer's solution containing the following (in mM): 126 NaCl, 1.2 NaHPO4, 2.4 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 10 glucose, and 18 $NaHCO_3$, pH 7.4. The holding potential was maintained at -70 mV to record Ach currents. Currents were filtered at 2 kHz and digitized at 10 kHz. Drugs were applied using a hand-held pipette and used at the following concentrations: 1 mM acetylcholine, 1 mM α-GPC, and 10 nM α-bungarotoxin.

Statistical Evaluation

All experiments were run in triplicate. Data were analyzed by the one-way ANOVA test, followed by the Bonferroni's post-hoc test. Statistical significance was set at a $p<0.05$.

RESULTS

1. Alpha-GPC reduces Aβ-induced toxicity in BV2 cells

To determine the effect of α-GPC on the BV2 cell viability, *in vitro* experiments were performed to analyze the impact of treatment with α-GPC on survival of BV2 microglial cells that underwent to Aβ toxicity, by MTT assay. In a first experiment, we tested the concentration-related (range 1 pM-100 μM) effect of α-GPC alone upon BV2 microglial cell viability following 24, 48 and 72 h. α-GPC did not significantly affect cell proliferation, nor it induced cell toxicity at most concentrations used. Nevertheless, at concentrations of 25, 50 and 100 μM α-GPC showed toxic effects, as demonstrated by a significant decrease of cell viability that were significant at all the time points studied (data not shown). Thus, we selected the concentrations of 1μM for the cell viability experiments. With the aim to explore possible protective effects of α-GPC on Aβ-related toxicity upon BV2 cells, cells were pre-treated for 1 h with α-GPC 1μM and treated for 24, 48 and 72 h with $A\beta_{1-42}$ (5μM). Our results demonstrate that α-GPC was able to mitigate $A\beta_{1-42}$-induced detrimental effect on BV2 microglial cells at all the time point studied (fig.**1**).

2. α-GPC reduces expression of Aβ-induced inflammatory molecules in BV2 microglial cells

To investigate whether the protective effect of α-GPC on Aβ treated BV2 was also related to change of microglia phenotype under Aβ-induced inflammation, the levels of phenotype-associated molecules were analyzed by immunofluorescence. BV2 cells were challenged with $A\beta_{1-42}$ (5 μM) alone or after 1 h pre-treatment with α-GPC 1 μ M for 48 h. The time point of 48 h was selected for the following experiments as it represents the time point in which inflammatory phenotypes were activated. As shown in Figure **2**, Aβ treatment strongly increased the expression of CD86 and TNF-α in BV2 cells, indicative of the M1 pro-inflammatory phenotype. In contrast α-GPC treatment prior to Aβ stimulation significantly counteract the Aβ-induced increase of CD86 and TNF-α (fig. 2). Consistently, Aβ treatment decreased the expression of the anti-inflammatory molecules CD68 marker and IL-10 whereas the expression of same molecules was increased in BV2 cells underwent Aβ treatment but pretreated with α-GPC (fig. **3**). To further confirm our observations, Western blot analysis (fig. **4**) for the expression of the inflammatory

cytokine TNF-α, as well as the anti-inflammatory cytokine IL-10, were performed on lysates of BV2 underwent the above-mentioned treatment. The expression of TNF-α was substantially increased in BV2 cells challenged with $A\beta_{1-42}$, whereas BV2 cells pre-treated with α-GPC showed a significant attenuation of its expression. On the other hand, the expression of IL-10 reduced in BV2 challenged with $A\beta_{1-42}$, was significantly increased when BV2 were pretreated with α-GPC. Densitometric analysis confirmed all these results (Fig. 4A', B'). These data demonstrate that α-GPC can induce a switch of Aβ -activated M1 microglia towards the M2 phenotype.

3. *α7 nAchR is expressed in BV2 cells and is modulated by α-GPC treatment*

It is well documented the expression of α7 nAChR in microglia BV2 cells (**Nakamura Y, 2020**) and that activation of microglial α7 nAChR suppresses the production of several proinflammatory molecules (**De Simone R, 2005**) (**Suzuki T, 2006**). To verify whether α-GPC treatment can affect α7 nAchR expression in microglia exposed to Aβ, we performed fluorescent immunocytochemistry in BV2 cells challenged for 48 h with $A\beta_{1-42}$ (5 μM) alone or after 1 h of pre-treatment with α-GPC 1 μM. Although BV2 cell cultures that underwent the different treatment express basal levels of α7 nAChR in, its expression was reduced in cells treated with Aβ and return to control levels when cells were pre-treated 1 h with α-GPC, as shown by the representative images (fig. **5**). Densitometric analysis of the fluorescence signal confirm such significant differences (fig. 5A). Consistently, Western blot analysis performed on BV2 cells lysates treated with α-GPC and/or $A\beta_{1-42}$ to verify the modulation of α7 nAChR expression corroborate previous data. (fig. **4**C, C'). These results confirm that α-GPC efficiently protect BV2 microglia cells from damage induced by Aβ by acting as an efficient modulator of the receptor α7 nAChR.

4. *Alpha-bungarotoxin, an antagonist of α7 nAChR, exacerbated Aβ-induced toxicity in BV2 cells.*

To corroborate the hypothesis that α-GPC acts as an efficient modulator of α7 nAChR, it was investigated whether α7 nAChR competitive antagonist α-bungarotoxin, may affect the protective effect of α-GPC on BV2 cells proliferation

after the challenge with $A\beta_{1-42}$, using MTT assay. Treatments with α-bungarotoxin (100 nM, α-BTX) for 24, 48 and 72 h were found to significantly reduce BV2 cells viability in the presence of $A\beta_{1-42}$ (5 µM), pre-treated with α-GPC (1 µM), at 72h (fig. **6**), suggesting the key role of α7 nAChR in mechanism of protection from Aβ-induced toxicity.

5. *Alpha-bungarotoxin prevented choline alphoscerate (a-GPC) effect on α7 nAChR-mediated $[Ca^{2+}]_i$ increase and inward currents in microglial cells*

Due to α7 nAChR involvement in cognition (**Leiser SC, 2009**), attention (**Hayward A, 2017**) and neuroprotection (**Dineley KT, 2015**); (**Deutsch SI, 2016**), we explored its functional involvement in acetylcholine response in microglial BV2 cells, analyzing intracellular calcium $[Ca^{2+}]$i levels either in the presence or absence of α-bungarotoxin (α-BTX). To demonstrate that the effects of α-GPC on microglia are mediated by the α7 nAChR, BV2 cells were challenged with Ach or α-GPC, alone and in combination with α-BTX. Results showed that Ach induced a significant increase in $[Ca^{2+}]_i$ in Fura2-loaded cells, an effect that was inhibited by α-BTX (Fig. **7**A, B, C, D). Furthermore, Ach induced a rapid inward current measured by patch-clamp electrophysiology that was quite completely inhibited by α-BTX. Such findings imply predominant role of α7 nAChR in microglial cells, in line with previous research (**Aripaka SS, 2021**). To assess the ability of α-GPC to directly activate α7 nAChR receptors, we tested its impact on Fura2-loaded BV2 cells in the presence or absence of α-BTX. Interestingly, α-GPC significantly increased $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig. **8**A). Moreover, α-BTX prevented α-GPC-induced effect on $[Ca^{2+}]_i$ (Fig. **8**B), suggesting its ability to selectively modulate α7 nAChR receptor in microglial cells. Similarly to Ach, α-GPC elicited an inward current measured by patch-clamp electrophysiology, and this effect was blocked by α-BTX (Fig. 8C, D). Furthermore, the α-GPC-induced current was found to be higher than that elicited by Ach, possibly due to a delayed current inactivation (Fig. 8E).

6. The α7 nAChR Antagonist, reversed the effects of α-GPC on anti-inflammatory signaling molecules

To assess the role of the α7 nAChR antagonist α-bungarotoxin in the regulation of the anti-inflammatory effects mediated by α-GPC, we performed experiments in BV2 cells challenged with the α7 nAChR antagonist (α-BTX), alone or in presence of α-GPC and/or $A\beta_{1-42}$, and the expression of proinflammatory molecules CD86 and TNF-α, as well as the anti-inflammatory molecules CD68 and IL-10 were evaluated by fluorescent immunocytochemistry. Results depicted in figs. **9-10** showed that BV2 underwent treatment with α-BTX inhibited the anti-inflammatory effects of α-GPC challenged along with $A\beta_{1-42}$. In fact, the expression of the inflammatory molecules was substantially increased in BV2 cells challenged α-BTX in the presence of $A\beta_{1-42}$ pre-treated with α-GPC (fig. **9**). On the other hand, the expression of IL-10 as well as CD68 was reduced in BV2 cells challenged with α-BTX in the presence of $A\beta_{1-42}$ pre-treated with α-GPC (fig. **10**). Densitometric analysis confirmed all these results (Figs. 9A-10A).

These findings suggest that α7 nAChR mediates α-GPC anti-inflammatory effects in microglia by modulating pro- and anti-inflammatory signaling molecules.

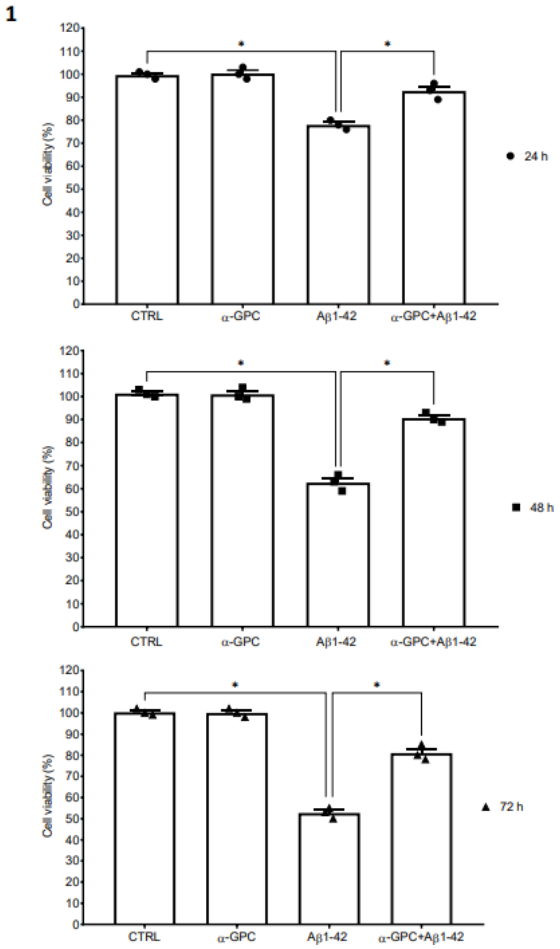

Figure 1: *α-GPC mitigates $A\beta_{1\text{-}42}$-induced detrimental effect on BV2.*

Cell viability (%) of BV2 cells pretreated for 1h with α-GPC (1μM) and treated for 24, 48, and 72h with $A\beta_{1\text{-}42}$ (5 μM). Vertical bars are means ± S.E.M. One-way ANOVA and the Bonferroni post-hoc test were used for statistical analysis. * $p < 0.05$.

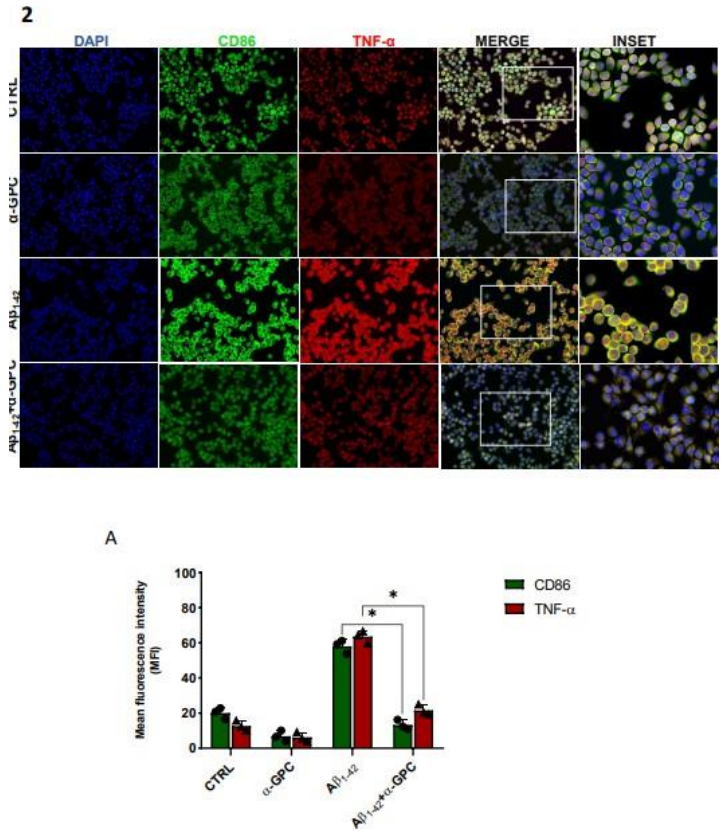

Figure 2: *Proinflammatory microglia is blunted by α-GPC treatment.*
Representative images (original magnification 20x; 40x) of the fluorescent immunocytochemical detection of CD86 and TNF-α expression in BV2 cells pretreated for 1h with α-GPC (1μM) and treated for 48h with $A\beta_{1\text{-}42}$ (5 μM). **A**) Respective densitometric analysis. Data are expressed as means ± S.E.M. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. * $p < 0.05$

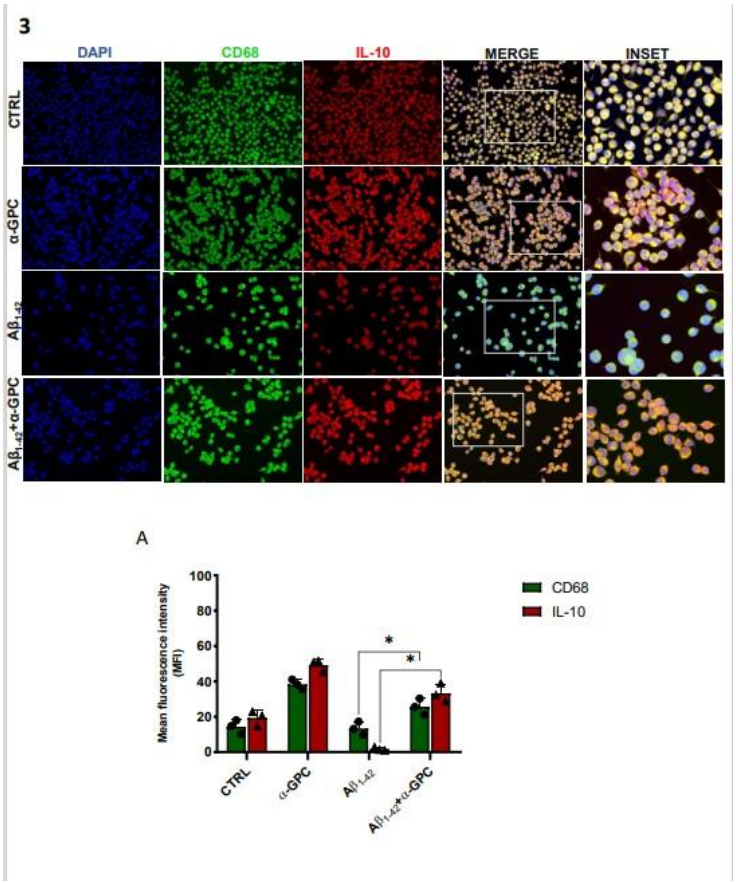

Figure 3: *α-GPC contributes to M2 phenotypic switching of microglia*.

Representative images (original magnification 20x; 40x) of the fluorescent immunocytochemical detection of CD68 and IL-10 expression in BV2 cells pretreated for 1h with α-GPC (1μM) and treated for 48h with $A\beta_{1\text{-}42}$ (5μM). **A)** Respective densitometric analysis. Data are expressed as means ± S.E.M. One-way ANOVA and the Bonferroni post-hoc test were used to determine statistical significance. * $p < 0.05$

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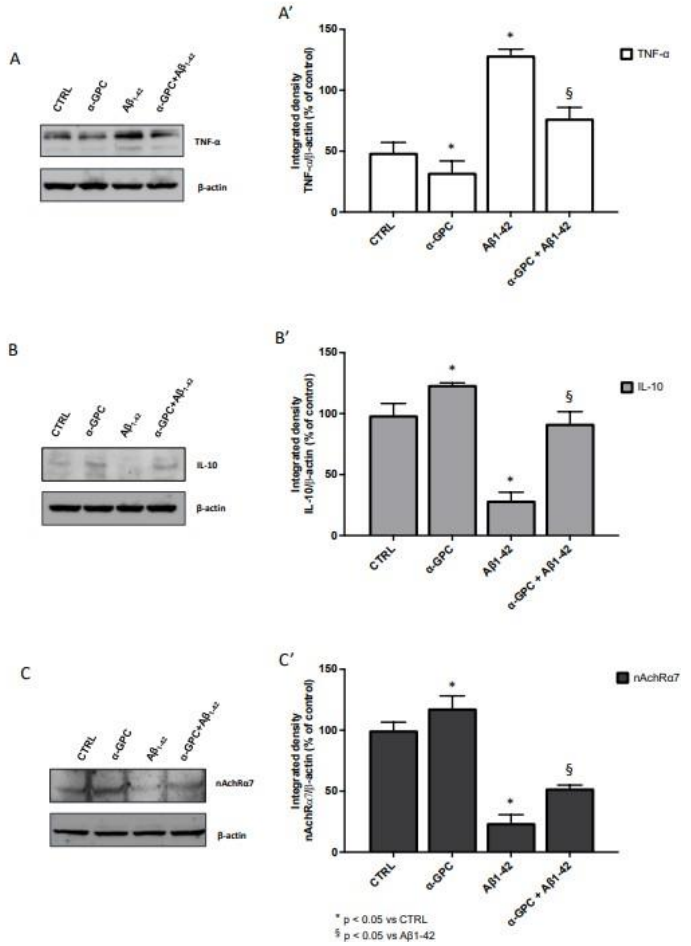

Figure 4: Western blot for TNF-α, IL-10 and α7 nAChR protein expression in BV2 cells and respective densitometric analysis (**A'**, **B'**, **C'**) of the representative Western blot (**A**, **B**, **C**). Data are expressed as means ± S.E.M. One-way ANOVA and the Bonferroni post-hoc test were used for statistical analysis. * $p < 0.05$.

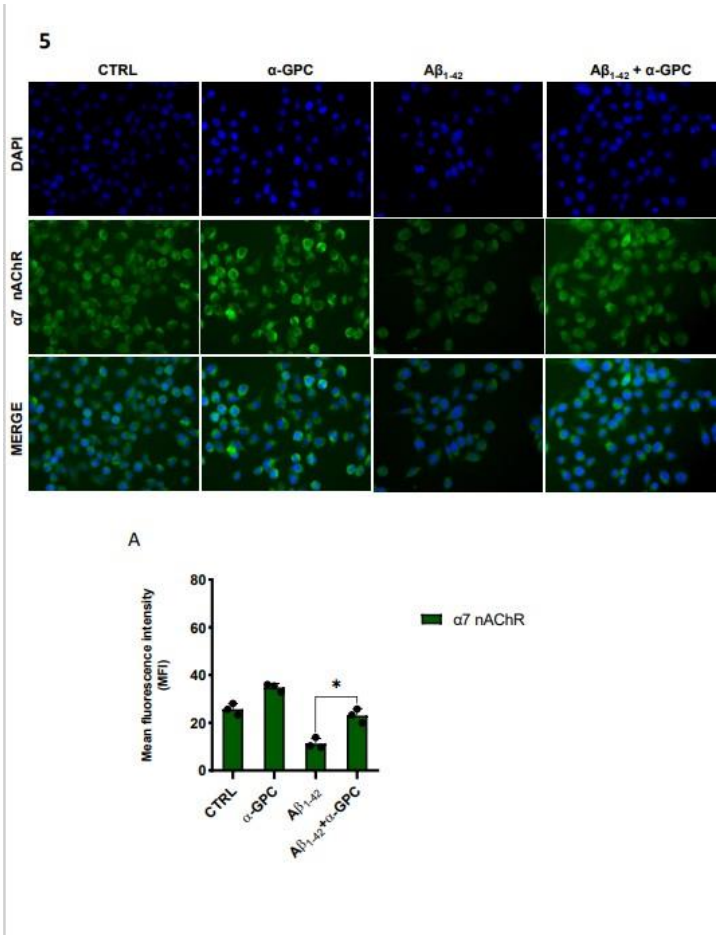

Figure 5: *α7 nAchR is modulated by α-GPC treatment.*

Fluorescent immunocytochemistry of α7 nAChR in BV2 cells pretreated with α-GPC (1μM) and treated for 48h with $A\beta_{1\text{-}42}$ (5μM) and respective densitometric analysis (**A**).

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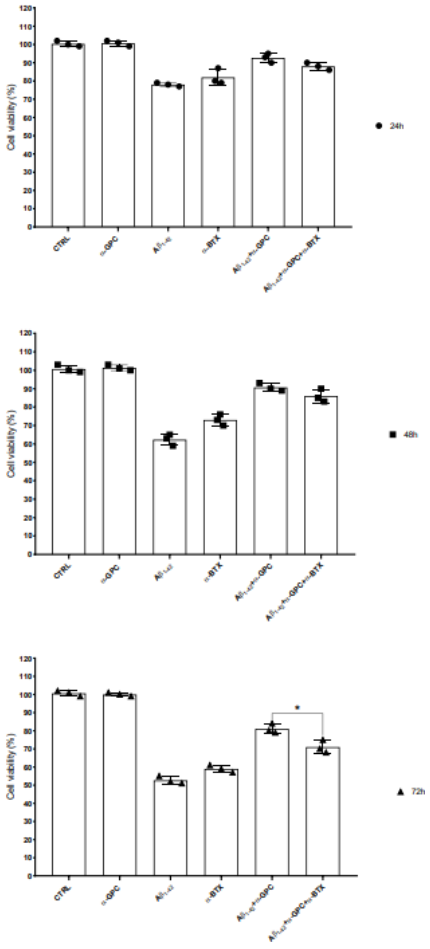

Figure 6: *α-bungarotoxin affects the protective effect of α-GPC on BV2 cells challenged with* $A\beta_{1\text{-}42}$ Cell viability (%) of BV2 cells pretreated for 1h with α-GPC (1μM) and treated for 24, 48 and 72h with $A\beta_{1\text{-}42}$ (5μM)/ α-BTX (100nM). Vertical bars are means ± S.E.M. One-way ANOVA and the Bonferroni post-hoc test were used for statistical analysis. * $p < 0.05$.

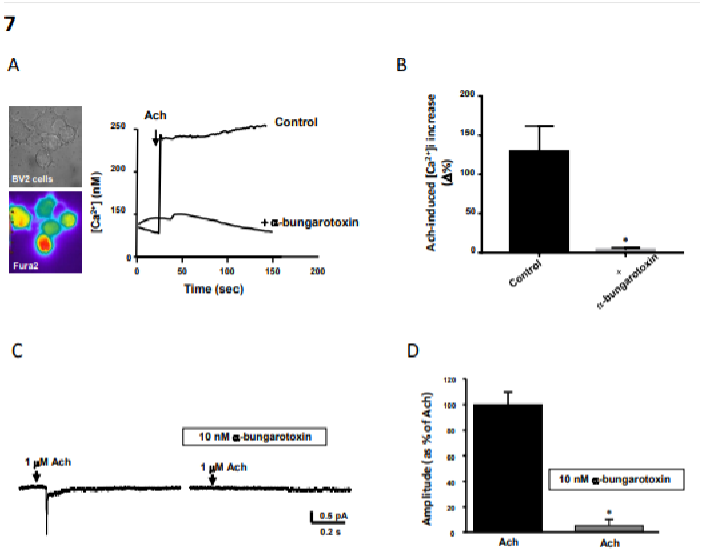

Figure 7: *Effect of a-bungarotoxin on acetylcholine -induced $[Ca^{2+}]_i$ increase and acetylcholine -induced inward current in microglial BV2 cells.*

A, **B** Representative traces and quantification for the effect of acetylcholine (Ach, 1μM) alone or in the presence of a-bungarotoxin (10nM) on $[Ca^{2+}]_i$, expressed as D% increase peak over basal values (N=40 cells for Ach and N=35 cells for Ach+α-bungarotoxin). On the left, representative brightfield and pseudocolor images of Fura-2 loaded BV2 cells. **C**, **D** Representative current traces in response to Ach (1μM) alone or in the presence of α-bungarotoxin (10nM), and quantification as current amplitude. For A-D, *p < 0.05 vs Ach 1μM.

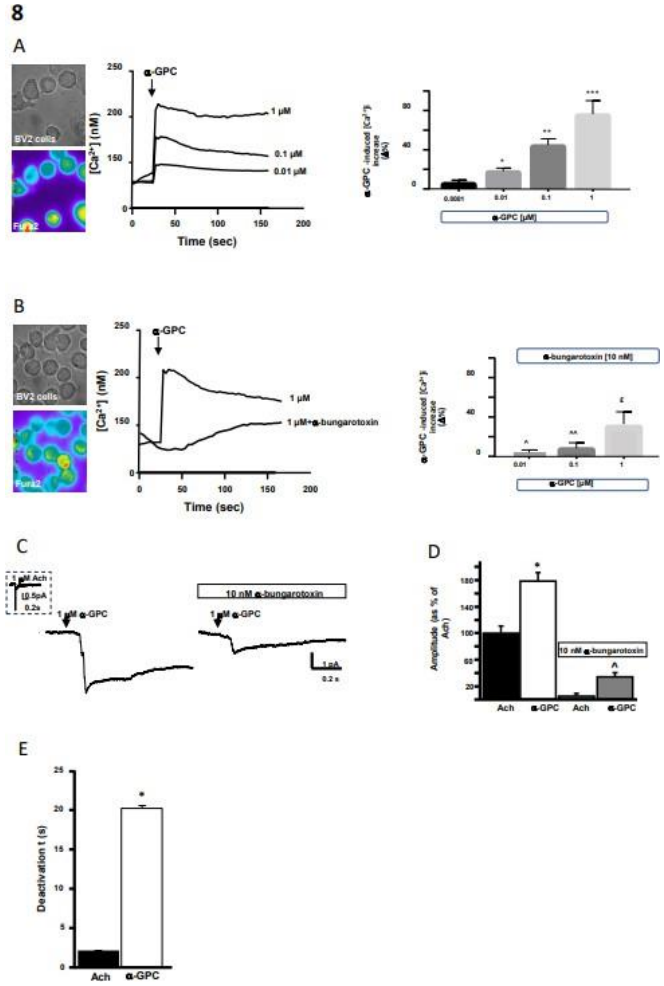

Figure 8: *Effect of α-GPC on $[Ca^{2+}]_i$ increase and α7 nAChR-encoded inward current in microglial BV2 cells*

A, **B** Representative traces and quantification for the effect of different concentrations of α-GPC (0.01-1μM) alone or α-GPC (1μM) +α-bungarotoxin (10nM) on $[Ca^{2+}]_i$, expressed as D% increase peak over basal values (N=35 cells for a-GPC and N=30 cells for α-GPC +α-bungarotoxin). On the left of each panel, representative brightfield and pseudocolor images of Fura-2 loaded BV2 cells. *p < 0.05 vs control (basal values) and 0.0001μM; **p<0.05 vs control and 0.01μM; ***p <0.05 vs control and 0.1μM. **C**, **D** Representative current traces in response to Ach (1 μM) alone or Ach+α-bungarotoxin (10 nM), and quantification as current amplitude. **E**. Representative higher α-GPC-induced current than that elicited by Ach.

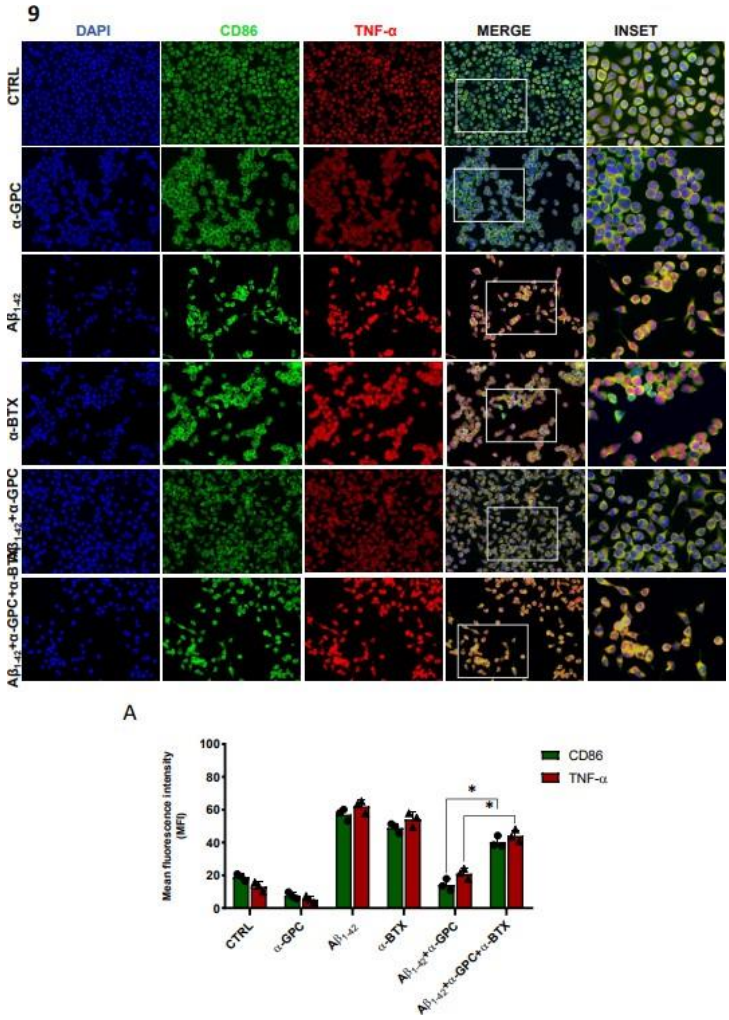

Figure 9: *α-BTX restores pro-inflammatory molecules expression.*

Representative images (original magnification 20x; 40x) of the fluorescent immunocytochemical detection of CD86 and TNF-α expression in BV2 cells pretreated for 1h with α-GPC (1μM) and treated for 48h with $A\beta_{1-42}$ (5 μM)/ α-BTX (100nM). **A)** Respective densitometric analysis. Data are expressed as means ± S.E.M. Differences between groups were considered significant at *p < 0.05 (One-way ANOVA followed by Bonferroni post-hoc test).

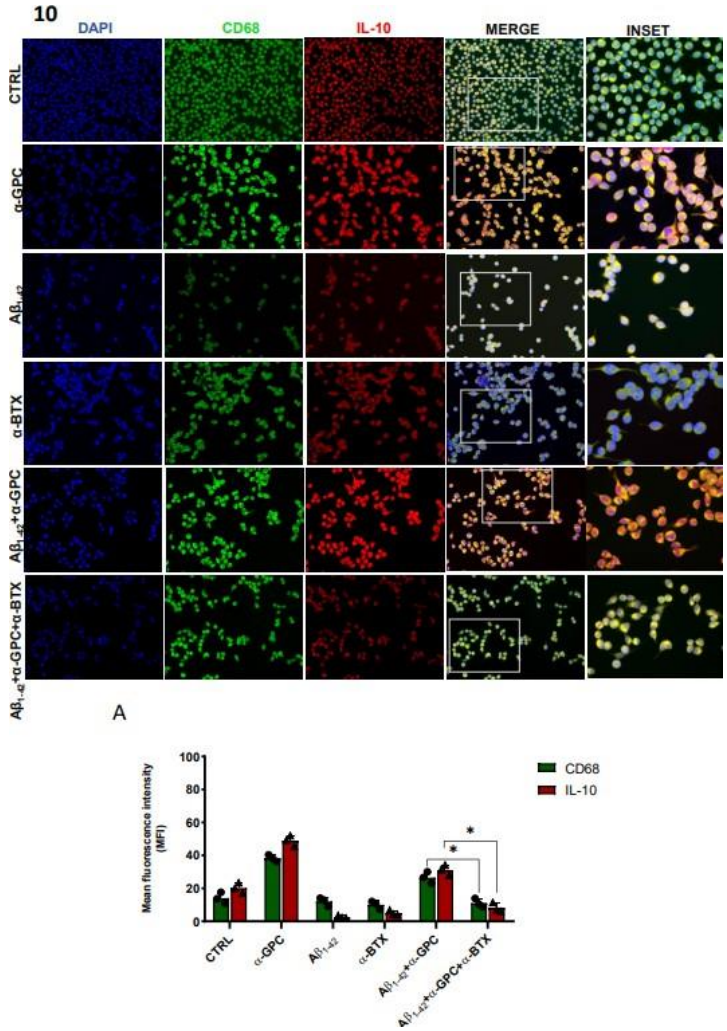

Figure 10: *α-BTX inhibits the anti-inflammatory effects of α-GPC*

Representative images (original magnification 20x; 40x) of the fluorescent immunocytochemical detection of CD68 and IL-10 expression in BV2 cells pretreated for 1h with α-GPC (1μM) and treated for 48h with $A\beta_{1\text{-}42}$ (5 μM)/ α-BTX (100nM). **A**) Respective densitometric analysis. Data are expressed as means ± S.E.M. Differences between groups were considered significant at *$p < 0.05$ (One-way ANOVA followed by Bonferroni post-hoc test).

DISCUSSION

In the present study, we investigated the effect of choline alphoscerate (α-GPC), a phosphatidylcholine derivative, as an effective agent for enhancing cholinergic transmission. We studied its impact on microglial phenotypic switching using an *in vitro* model of BV2 culture cells treated with $A\beta_{1-42}$, while also examining the involvement of α7 nAChR in this effect. We first assessed the impact of α-GPC on BV2 cells viability and its potential protective role against Aβ-related toxicity in the same cell line. We showed that at different concentrations, α-GPC did not interfere with cells proliferation and/or survival, while it effectively mitigates $A\beta_{1-42}$-induced detrimental effect in BV2 microglial cells at all the time point studied.

Previous works demonstrated that α-GPC preserved neuronal cells from Aβ-induced toxicity **(Catanesi M, 2020); (Burgaletto, 2021)** suggesting the possibility of a similar protective effect on BV2 treated with $A\beta_{1-42}$. Similar to Ach, α-GPC, which contains choline in its structure and may act as a precursor to Ach, can modulate microglial activity in response to Aβ. Such modulation may involve changes in cytokines release, phagocytic activity, and other microglial functions (**Tayebati, 2015**). In light of the growing body of evidence supporting the concept of the "cholinergic anti-inflammatory pathway" and its relevance to both immunity and neuroinflammation (**Pavlov VA, 2003**), and considering the potential protective role of α-GPC, particularly in the context of neuroinflammation, we investigated the effect of α-GPC on microglial phenotypes in response to Aβ insult. Our data demonstrate that α-GPC induces a shift of Aβ -activated M1 microglia to the M2 protective phenotype. Indeed, BV2 microglial cells challenged with $A\beta_{1-42}$ exhibited a pro-inflammatory M1 status, characterized by high expression of CD86 and TNF-α. On the other hand, treatment with $A\beta_{1-42}$ in the presence of α-GPC induced a phenotypic switch to M2 status, distinguished by a significant attenuation of the expression of the inflammatory markers. Furthermore, the reduced expression of the anti-inflammatory IL-10 in BV2 challenged with $A\beta_{1-42}$ was significantly increased when BV2 were pre-treated with α-GPC.

In addition, the role of the "cholinergic anti-inflammatory pathway" in immunity and neuroinflammation has received considerable attention, as alterations in α7 nAChRs have been correlated with several pathologies, including Alzheimer's disease (**Piovesana R, 2021**).

The activity of α7 nAChRs expressed by glial cells may counterbalance the

neuroinflammatory effects of Aβ fragments. (**Takata K, 2018**). Previous in vitro studies have reported that activation of α7 nAChRs with a selective agonist promotes Aβ phagocytosis by cultured microglial cells (**Takata K, 2018)**. On the other hand, in vivo studies in an AD mouse model have also shown that α7 nAChR stimulation improves cognitive function (**Medeiros R, 2014**). It has further been reported that BV2 cells express mRNA for the α7 subunit of nAChRs. (**Aripaka SS, 2020**)

It is well established that nicotinic receptors have different permeabilities to calcium ions, with the homomeric α7 subtype exhibiting one of the highest Ca2+: Na+ permeabilities ratio (**Fucile S, 2003**). Given the plethora of significant cellular pathways induced by calcium influx through this receptor, particularly those involved in neuroprotection, we performed [Ca2+]i measurement (**Shen JX, 2009**) (**Fucile S, 2004**).

More specifically, considering α7 nAChR involvement in cognition (**Leiser et al., 2009**), attention (**Hayward A, 2017**) and neuroprotection (**Dineley et al., 2015**) ; (**Deutsch et al., 2016**), its functional role in Ach response in microglial BV2 cells in the presence or absence of the specific inhibitor α-bungarotoxin (α-BTX), was investigated. In this respect, Ach induced a significant increase in [Ca2+]i in Fura2-loaded cells, which was prevented by α-BTX. Furthermore, Ach induced a fast inward current as measured by patch-clamp electrophysiology, which was quite completely inhibited by α-BTX. These data suggest a functional predominance of the α7 nAChR in microglial cells, as previously reported (**Aripaka SS, 2020).**

To assess its ability to directly activate α7 nAChR receptors, α-GPC activity was tested on Fura2-loaded BV2 cells in the presence or absence of α-BTX. Accordingly, α-GPC significantly increased [Ca2+]i in a concentration-dependent manner. Furthermore, α-bungarotoxin prevented this effect on [Ca2+]i, suggesting the ability of α-GPC to selectively modulate the α7 nAChR receptor in microglial cells. In addition, α-GPC elicited an inward current measured by patch-clamp electrophysiology that was inhibited by a-bungarotoxin. Both responses are similar to Ach, but interestingly the α-GPC-induced current was higher than that elicited by Ach, possibly due to a delay in current inactivation.

To further support the hypothesis that α7 nAChR mediates the anti-inflammatory

effects of α-GPC, we demonstrated that treatment of BV2 with α-BTX prevented the anti-inflammatory effects of α-GPC in the presence of $A\beta_{1-42}$, as evidenced by the significantly increased expression of TNF-α and CD86 in these cells. In contrast, levels of IL-10 and CD68 were reduced. These data are consistent with previous studies showing that the choline-mediated anti-inflammatory effect on microglial activation through α7 nicotinic receptors is reversed by the selective α7 nicotinic receptor antagonist α-bungarotoxin (**Shytle RD, 2004**)

Our findings demonstrate that α-GPC has the ability to induce a switch in microglial phenotype from the M1 to the M2 state mediated by nAChR α7, in an in vitro model of AD.

Overall, the study results suggest that α-GPC may have an anti-inflammatory role in AD, possibly exerting its beneficial effects by enhancing cholinergic stimuli that could antagonise microglial-mediated inflammation, activating the α7 nAChR system. In view of the above, it is plausible that therapeutic use of α-GPC may be a promising strategy to reduce neuroinflammation, protect neurons and attenuate AD pathology.

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Chapter 2

BENEFICIAL EFFECTS OF CHOLINE ALPHOSCERATE IN THE 3×TG-AD MOUSE MODEL OF ALZHEIMER'S DISEASE

Beneficial effects of choline alphoscerate in the 3xTg-AD mouse model of Alzheimer's Disease.

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ABSTRACT

Background: Alzheimer's disease (AD) is the most common form of dementia, characterized by progressive degeneration and loss of neurons in specific regions of the central nervous system. Chronic activation of the immune cells resident in the brain and release of inflammatory and neurotoxic factors appear critical contributors of the neuroinflammatory response in AD. Considering the disruption in cholinergic neurotransmission in the disease, the purpose of this study is to investigate the effects of alpha-glyceryl phosphorylcholine (α-GPC), a cholinergic enhancing molecule, in a triple transgenic mouse model of AD (3xTg-AD).

Methods: 3xTg-AD mice were chronically treated with α-GPC for 8 months to evaluate its effects upon neuroinflammation, synaptic function and cognitive performance.

Results: Treatment with α-GPC led to a substantial rebalance of the inflammatory response of resident innate immune cells, astrocytes and microglia. In fact, α-GPC contributes to reduction of reactive astrocytes, expressing GFAP and iNOS markers, as well as pro inflammatory microglia expressing Iba1 and TNF-α. On the other hand, α-GPC increases the expression of anti-inflammatory IL-10 expressing microglia and of the synaptic marker synaptophysin in the hippocampus.

Furthermore, we observed that 3xTg-AD mice treated with α-GPC significantly spent more time exploring the novel object (NOR test).

Conclusions: Chronic treatment with α-GPC demonstrate a significant anti-inflammatory activity and sustain the key function of hippocampal synapses crucial for the maintenance of a regular cognitive status. Therefore, we suggest that α-GPC could be exploited as a promising therapeutic approach in very early phases of AD.

Keywords: Alzheimer's Disease, Neurodegeneration, Inflammation, Immune response.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder representing the major cause of dementia worldwide, characterized by an irreversible decline in episodic memory and then a more general deterioration in overall cognitive ability [1]. This devastating condition is pathologically underpinned by the accumulation of extracellular amyloid-β (Aβ) plaques and intracellular neurofibrillary tangles of hyperphosphorylated tau protein, resulting in synaptic and neuronal loss [2]. Additionally, neuroinflammation plays a significant role in the onset and development of AD-related neurodegeneration [3].

The well-known and selective degeneration of basal forebrain cholinergic neurons and the subsequent dysfunction of cholinergic transmission have long been deemed as driving factors the disease development and therefore have steadily directed the main therapeutic efforts in the drug discovery process [4]. Cholinergic precursors have represented one of the first approaches attempting to counteract cholinergic impairment and to relieve cognitive decline occurring in dementia disorders. Among these, choline alphoscerate, also known as alpha-glycerophosphocholine (α-GPC), a semisynthetic derivate of lecithin, is considered one of the most suitable sources of choline, an essential nutrient and precursor of the neurotransmitter acetylcholine (Ach) [5]. Indeed, α-GPC, encompassing choline in its structure, seems to have a significant effect on enhancing Ach synthesis and release, due to its ability to cross the blood-brain-barrier (BBB) and reach the central nervous system (CNS), contributing to neuronal membranes reorganization [6].

Several preclinical studies have shown that α-GPC promotes learning and memory in experimental brain aging models, by improving brain transduction mechanisms, and preventing age-dependent structural changes occurring in crucial areas such as frontal cortex and hippocampus [7]. Moreover, α-GPC has been proven effective to reverse mnemonic deficits induced by scopolamine administration, suggesting a specific increase of hippocampal Ach synthesis and release [8,9]. Although beneficial effects of α-GPC have been extensively reported in experimental models, only sparse research have assessed the mechanisms underlying such effects. Recently, in vitro experiments performed in the SH-SY5Y human cell line have revealed the α-GPC helpful role upon Aβ toxicity by setting into motion neurotrophins- signaling pathways, known to be knocked down in AD, also

sustaining the expression level of synaptic proteins related to neuronal survival [10].

Compelling evidence suggest that a robust glial-mediated inflammatory response manifests as an early feature of AD pathophysiological mechanisms, playing a pivotal role [11]. Glial cells, especially microglia and astrocytes, engaging a fine-tuned crosstalk, perform housekeeping functions essential to neuronal health. However, a detrimental sustained inflammatory response trapped glia-mediated mechanisms in a vicious cycle fueling neurodegeneration [12]. Notably, glial nicotinic acetylcholine receptors (nAchRs) have a role in different AD-related phenomena, including Aβ degradation, synaptic plasticity, and memory. Specifically, these receptors suppress glial pro- inflammatory cytokines production, enhance Aβ phagocytosis and induce oxidative stress suppression [13]. Therefore, increasing Ach synthesis and release could counteract these unwholesome issues by reducing neuroinflammation and improving neurological outcomes.

With such rationale, and with the purpose to better highlight the mechanisms underlying the beneficial effects of α-GPC in AD, herein we investigate whether the chronic treatment with α-GPC is related to an immune rebalance, and whether this could correlate with an improvement of the cognitive outcome in a triple transgenic mouse model of AD (3xtg-AD). To achieve this task, we used a mouse model which develops both plaques and tangles in an age-related fashion in hippocampal and cortical regions, paralleled by learning and memory impairment [14]. Mice were treated chronically for 8 months to evaluate the effects of α-GPC supplementation, upon parameters of neuroinflammation, synaptic function, as well as upon cognitive performance.

MATERIALS AND METHODS

Animals

Male 3xTg-AD mice [B6129-Psen1tm1MpmTg (APPSwe, tauP30L) 1Lfa/J] [14] and wild-type mice (B6129SF2/J) were purchased from Jackson Laboratories. The 3xTg-AD, overexpressing mutant APP (APPSwe), PSEN1 (PS1M146V), and hyperphosphorylated tau (tauP301L), were originally generated by co-injecting two independent transgene constructs encoding human APPSwe and tauP301L (4R/0 N) (controlled by murine Thy1.2 regulatory elements) in single-cell embryos harvested from mutant homozygous PS1M146V knock-in mice. Wild-type mice of mixed genetic background 129/C57BL6 were used as controls. These mice, well-characterized and described by Oddo et al. [14]. The mice were maintained on a 12-h light/dark cycle in temperature and humidity-controlled rooms, and food and water were available ad libitum. All experiments were carried out according to the Directive 2010/63/EU and the Italian law (D.Lgs. 26/2014) and were approved by the Italian Ministry of Health.

Drug administration and experimental groups

Twenty-two 3xTg-AD and twenty-two wild-type mice were enrolled at 4 months of age and four study groups were used: (I) wild-type plus vehicle; (II) wild-type plus 100 mg/kg/day α-GPC; (III) 3xTg-AD plus vehicle; (IV) 3xTg-AD plus 100 mg/kg/day α-GPC. Animals belonging to the second and fourth group received α-GPC dissolved in drinking water at a concentration resulting in an average daily dose of 100 mg/kg according to the procedure detailed elsewhere [15]. After 8 months of treatment (at 12 months of age) animals were sacrificed.

Novel object recognition (NOR) test

The NOR test was performed as previously described with minor modifications [16]. The behavioral test was performed in regularly illuminated (40 ± 1 lux) grey open fields (44 x 44 x 40 cm, Ugo Basile, Gemonio, Italy). The objects were different in shape, color and size (4 x 4 x 4 cm to 6 x 6 x 6 cm). They were fixed to the floor of the apparatus to circumvent displacements during the test. The researchers handled animals on alternate days during the week preceding the stress procedure. Animals were acclimatized to the testing room 1 h before the beginning of the tests A 2-day pretest was performed to acclimatize mice to the apparatus as well as to prevent neophobia during the test. Mice were placed into the empty apparatus and allowed to freely explore for 15 min on day 1. Mice were instead allowed to explore the apparatus with two objects (different from those eventually used during the test) for 10 min during the day 2. The objects were placed in two corners of the apparatus, 10 cm far from the side walls. The test consisted of one sample phase and one test phase interspersed with 24- h delay in order to assess long-term recognition memory. During the sample phase (day 3), animals were placed in the center of the apparatus and allowed to explore two identical copies of an object for a total of 10 min. During the test phase, mice were allowed to explore for 10 min a copy of the familiar object previously explored in the sample phase, and a novel object never encountered. Mice performing a total exploration of the objects below 5 sec were excluded from the analysis. If the long-term recognition memory is intact, mice typically explore more the novel object rather than the familiar object. Cognitive performance during the test session was showed using the discrimination index (DI), calculated using the following formula: [(time spent exploring the novel object – time spent exploring the familiar object)/ total exploration time. The percentage of exploration of each object during the test session were also quantified. Behavioral experiments were carried out, recorded and analyzed by two expert researchers. The exploration of the objects were manually scored by the researchers. Each open field was cleaned with a 20% ethanol solution between sessions to minimize the impact of olfactory cues. A 12h light/12h dark cycle with was used. All behavioral experiments were performed during the light phase (9.00 a.m. to 4.00 p.m.).

Protein extraction

Tissues were lysed in a lysis buffer containing 150 manacle, 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1 mM Na3VO4, 30 mM sodium pyrophosphate, 50 mM NaF, 1 mM acid phenyl-methyl-sulphonyl- fluoride, 5 mg/ml aprotinin, 2 mg/ml leupeptin, 1 mg/ml pepstatin, 10% glycerol, and 0.2% TritonTM X-100. The homogenates were then centrifuged at 14 000 rpm for 10 min at 4C. The protein concentration of the supernatant was determined by the Bradford method (Bradford, 1976).

Western Blot Analysis

Equal amounts of proteins (50 µg) were separated by 8–12% SDS- PAGE gels and transferred onto Hybond ECL nitrocellulose membranes (Amersham Life Science, Buckinghamshire, UK). The membranes were blocked with 5% non-fat dry milk in PBST for 1 h at RT and were then probed overnight at 4 °C on orbital shaker with the following appropriate primary antibodies: mouse anti-synaptophysin (1:500; Abcam), goat anti-Iba-1 (1:1000; Novus Biologicals), mouse anti- GFAP (1:500; Cell Signaling Technology), rabbit anti-iNOS (1:500; Santa Cruz Biotechnology Inc.), rabbit anti-IL-10 (1:200, Abbiotec), rabbit anti-TNF-α (1:1000; Novus Biologicals). mouse anti-Beta-Actin (1:1000; Santa Cruz Biotechnology Inc.) primary antibody was used as an internal control to validate the right amount of protein loaded in the gels. Then the membranes were washed with PBS-T and probed with the appropriate horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for 1 h at room temperature in 5% non-fat dry milk. After washing with PBS-T, protein bands were visualized by enhanced chemiluminescence (Thermo Fisher Scientific) and scanned with the iBright FL1500 Imaging System (Thermo Fisher Scientific). Densitometric analysis of band intensity was performed with the aid of ImageJ software (developed by NIH, freeware, available online: https://imagej.nih.gov/ij/, accessed on 25 July 2022).

Immunofluorescence

Brain tissue specimens were fixed overnight in 10% neutral-buffered formalin (Bio-Optica). After overnight washing, they were dehydrated in graded ethanol and paraffin-embedded taking care to preserve their anatomical orientation. Tissue sections of 5μm were then cut and mounted on silanized glass slides and air dried. To remove the paraffin, slides were immersed in xylene two times, for 10 min each; rehydrated with graded ethanol, 100%, 95%, 70%, and 50%, two times for 10 min each; and transferred to distilled water. Antigens were retrieved in sodium citrate buffer (10 mM sodium cittrate, 0.05% Tween-20, pH 6.0) by microwave for 10 min, followed by rinsing with distilled water. The slides were then washed in PBS containing 0.025% Tween-20 (PBST) twice for 5 min each, blocked in 5% BSA/0.3% PBST for 1 h at room temperature, in humid chamber, and incubated at 4 °C overnight with BSA 1% and the following primary antibodies: goat anti-Iba1 antibody (Novus Biologicals, NB100-1028; 1:100) or a rabbit anti-IL10 antibody (Abbiotec, 250713; 1:200) or a rabbit anti-GFAP antibody (Dako, Z0334; 1:500), or a mouse anti-NOS2 antibody (Santa Cruz, sc-7271; 1:250) or a rabbit anti-TNFα antibody (Novus Biologicals, NB600-587; 1:100) or a mouse anti-synaptophysin antibody (Abcam, ab8049; 1:100). Antibodies were applied directly onto sections before overnight slide incubation (4 °C) in a humid chamber. For immunopositive reactions and fluorescence detection, after washing in PBST three times for 5 min each, sections were incubated with the corresponding fluorescent-labeled secondary antibodies in the dark for 1 h at room temperature: Alexa Fluor 546 donkey anti-goat IgG (Invitrogen; Thermo Fisher Scientific, Inc, MA, USA) or Alexa Fluor 488 donkey anti-rabbit (Thermo Fisher Scientific,) or Alexa Fluor 488 donkey anti- mouse (Thermo Fisher Scientific,) or Alexa Fluor 546 donkey anti-mouse (Thermo Fisher Scientific,) or Alexa Fluor 488 donkey anti- mouse (Thermo Fisher Scientific,). Finally, for staining of nuclei and stabilization of fluorescent signals, slides were covered in mounting medium (Fluoroshield with DAPI; Sigma-Aldrich, Milan, Italy) and secured with a coverslip.

Statistical analysis

Data were analyzed either by the one-way or the two-way analysis of variance (ANOVA), followed, respectively, by the Duncan's least significant difference test or by Bonferroni post-hoc test. Vertical bars are means ± S.E.M. of at least three different experiments; significance was set at a p value < 0.05 or $p < 0.001$. The graphs were made using Graph Pad Prism (Ver. 7, La Jolla, USA). Statistical evaluation was performed using standard computer software (SPSS software package, ver. 23.0, SPSS Inc., Chicago, IL, USA).

RESULTS

Chronic treatment with α-GPC is associated with reduction of gliosis in 3xTg-AD mice

Reactive gliosis is considered a key abnormality in neurodegenerative diseases, representing one of the most important pro-inflammatory mechanisms in AD neuropathology. Already detectable in the early stages of AD, becomes ubiquitous throughout disease progression [17]. In general, astrocyte reactivity has been defined as an increase in intermediate filaments, such as glial fibrillary acidic protein (GFAP), and by the risen expression of inducible nitric oxide synthase (iNOS), contributing to nitric oxide-mediated neurodegeneration in neighboring neurons [18]. Therefore, with the aim to verify whether reactive gliosis features were increased in 3xTg-AD mice, and whether the expression of its specific markers could eventually change in various treatment groups, we double-stained tissues with antibodies against GFAP and iNOS. Double immunofluorescent labeling images revealed that 3xTg- AD mice showed a broad astrocytic activation, paralleled by the increased expression of GFAP, co-localized with iNOS, in both the cortex and the hippocampus, as compared with WT mice (**Figure 1**). Notably, the expression of GFAP was dramatically decreased in animals that received the α-GPC treatment. Immunohistochemical data were consistent with those obtained by western blot analysis of hippocampal and cortical lysates from the same groups of animals, corroborating the significative reduction of activation markers expression in α-GPC treated animals (**Figure 5A; Figure 6A**).

α-GPC reduces the expression of proinflammatory markers in 3xTg- AD mice and blunts microglia activation

Microglia housekeeping functions are essential to brain health [19]. In contrast, chronic overactivation of microglia, which occurs in AD, causes brain inflammation leading to neuronal death [20]. In order to assess whether chronic α-GPC treatment can reduce the levels of activated microglia, we double-stained tissues with antibodies against Iba1, a well-known marker of microglia activation, and TNF-α, a pro- inflammatory cytokine.

Double immunofluorescent labeling images revealed that 3xTg-AD mice showed a widespread glia activation, paralleled by the increase in microglial Iba1, co-

localized with TNF-α, in both the cortex and the hippocampus, as compared with WT mice. Of note, chronic treatment with α-GPC markedly ameliorated microgliosis in such brain areas in 3xTg-AD mice (**Figure 2**). These data were consistent with those obtained by western blot and eventually revealed that Iba1-positive cells were significantly decreased in animals treated with α-GPC when compared with untreated AD mice. In addition, western blot analysis also indicated that the substantial TNF-α expression detected in the cortex and in the hippocampus of 3xTg-AD mice is reduced after the α- GPC treatment (**Figure 5B; 6B**).

Anti-inflammatory cytokine IL-10 release is rescued by α-GPC treatment in 3xTg-AD mice

Several evidence suggest that the expression of inhibitory anti- inflammatory cytokines, such as IL-10, may have beneficial effects on the regulation of AD-related neuroinflammation [21]. For instance, IL- 10, typically reduced in AD patients [22], causes a dose-dependent inhibition of the IL-6 secretion induced by Aβ in glial cells [23]. To further establish whether functional changes observed could be related to a neuroinflammatory process in the 3xTg-AD mice brain, and to explore the effect of α-GPC treatment, the IL-10 expression was qualitatively analyzed by fluorescent immunocytochemistry and measured by means of western blot analysis of hippocampal and cortical lysates from the same groups of animals. Immunofluorescence experiments revealed that Iba1 was abundantly expressed in untreated 3xTg-AD mice along with a dampened expression of IL-10. On the other hand, IL-10 levels were significantly increased in α-GPC treated animals (**Figure 3**). Consistently, Western blot analysis showed that, while the expression of the anti-inflammatory cytokine IL-10 was absent in the cortex of WT and 3xTg-AD mice, it became detectable in animals undergone the α-GPC treatment. Likewise, the severe reduction of hippocampal IL-10 levels of 3xTg-AD mice was rescued by the treatment (**Figure 5B; 6B**).

Effect of α-GPC treatment on neuronal plasticity in 3xTg-AD mice

Synapses are the fundamental units of information transfer and memory storage in the brain [24]. Several studies of AD brain and transgenic animal models have both shown how a defective synaptic transmission strongly correlates with cognitive decline [25]. Specifically, 3xTg-AD mice displayed localized neurodegeneration, synaptic impairment, and cognitive deficits by 6 months of age [26]. Synaptophysin, an abundant pre-synaptic glycoprotein, is regarded as a truthful index of neuronal synaptic density, it is indeed involved in different processes, including the vesicle trafficking machinery and synapse formation [27]. We therefore investigated whether α-GPC can restore neuronal plasticity in 3xTg-AD mice by assessing synaptophysin expression in the hippocampus and the cortex. As displayed in (**Figure 4**), the results of immunofluorescence staining showed a substantial decrease of synaptophysin immunoreactivity in untreated 3xTg-AD mice when compared to WT, and a clear recovery to levels similar as WT controls with α-GPC chronic treatment. Western blotting results also showed a clear decline of synaptophysin in the hippocampus of untreated 3xTg- AD mice with respect to controls (**Figure 5C**). Importantly, α-GPC treatment significantly reversed the down-regulated expression of synaptophysin in 3xTg-AD mice. On the other hand, no significant changes in cortical levels expressions were detected (**Figure 6C**). Hence, absence of synaptophysin alteration in 3xTg-AD mice is consistent with report on other animal model of AD [28,29] and suggest that the cognitive improvement shown above was not related to a massive change in synapses number.

Chronic treatment with α-GPC rescued the long-term recognition memory deficits of 3xTg-AD mice

Episodic memory deficits are one the most significant functional alterations in patients suffering from AD [30]. In this regards, 3xTg-AD mice represent a well-established transgenic model useful to study episodic memory deficits [31]. Here we tested the hypothesis that a chronic treatment with α-GPC could rescue the episodic-like memory deficits showed by 3xTg-AD mice in the Novel Object Recognition (NOR) test (**Figure 7A**). Analysis of the DI revealed that 3xTg-AD mice treated with α-GPC significantly discriminated between the familiar object and the novel object, while 3xTg-AD mice treated with vehicle did not (**Figure 7B**; Treatment: $F_{(1, 15)} = 16.03$; $P = 0.0012$; Treatment x Genotype: $F_{(1, 15)} = 10.99$; $P = 0.0047$). Indeed, 3xTg-AD mice treated with α-GPC significantly spent more time exploring the novel object while 3xTg-AD mice treated with vehicle spent approximately the same amount of time exploring both objects (**Figure 7C**; Object: $F_{(1, 30)} = 101.8$; $P < 0.0001$; Object x treatment: $F_{(1, 30)} = 32.06$; $P < 0.0001$; Object x genotype: $F_{(1, 30)} = 5.233$; $P = 0.0294$;

Object x treatment x genotype: $F_{(1, 30)} = 21.97$; $P < 0.0001$). Both WT mice treated with vehicle and WT mice treated with α-GPC showed an intact long-term recognition memory exploring more the novel object rather than the familiar object (**Figure 7B-C**).

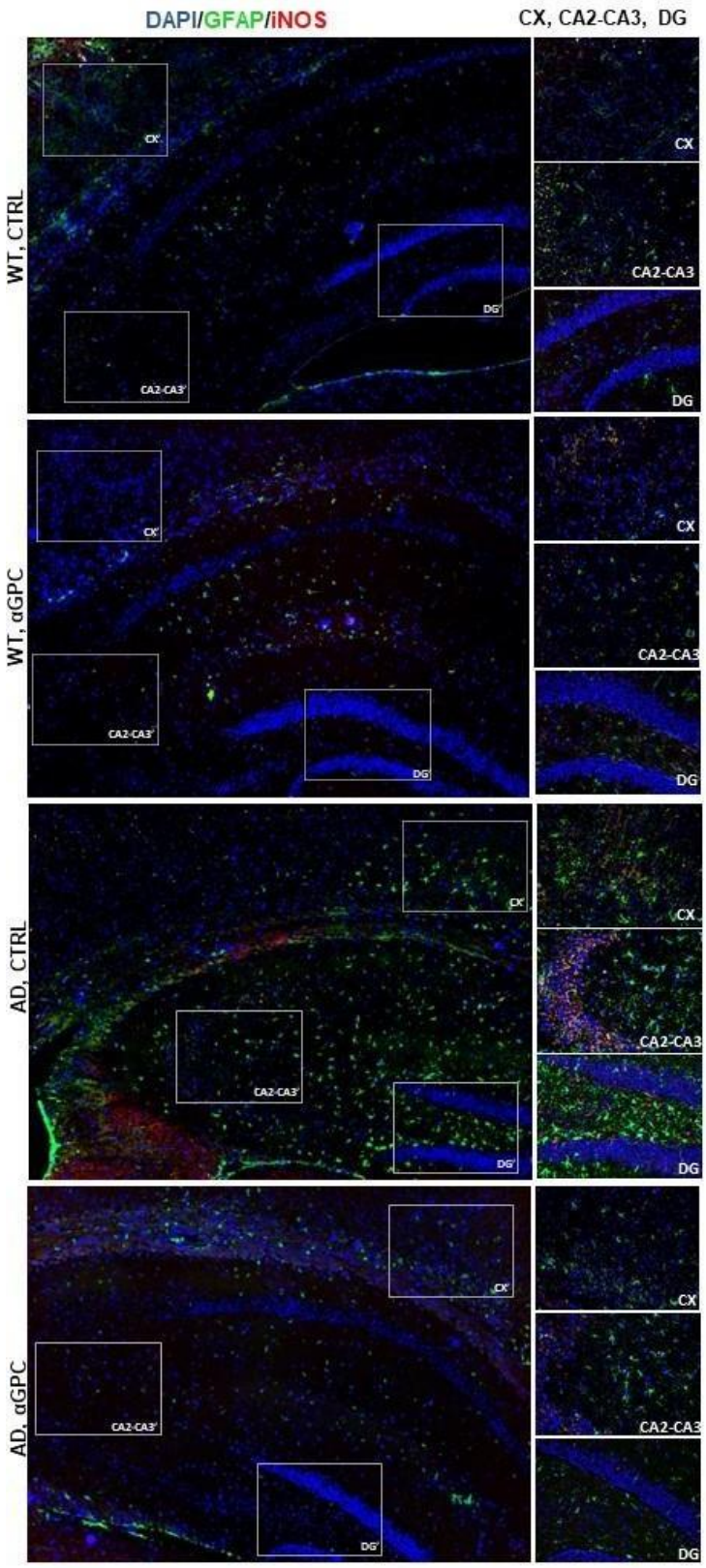

Figure 1: Chronic treatment with α-GPC is associated with reduction of gliosis in 3xTg-AD mice.

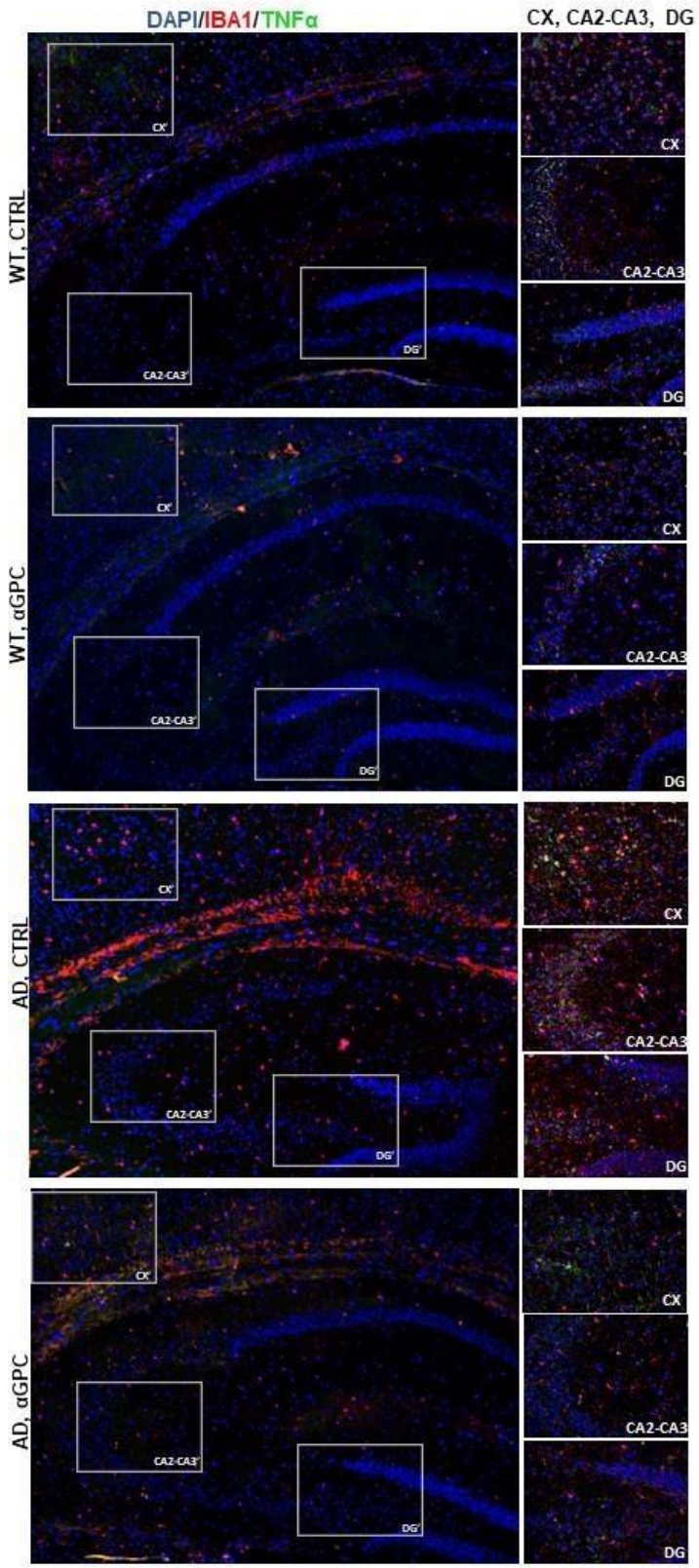

Figure 2: α-GPC reduces the expression of proinflammatory markers in 3xTg-AD mice and blunts microglia activation.

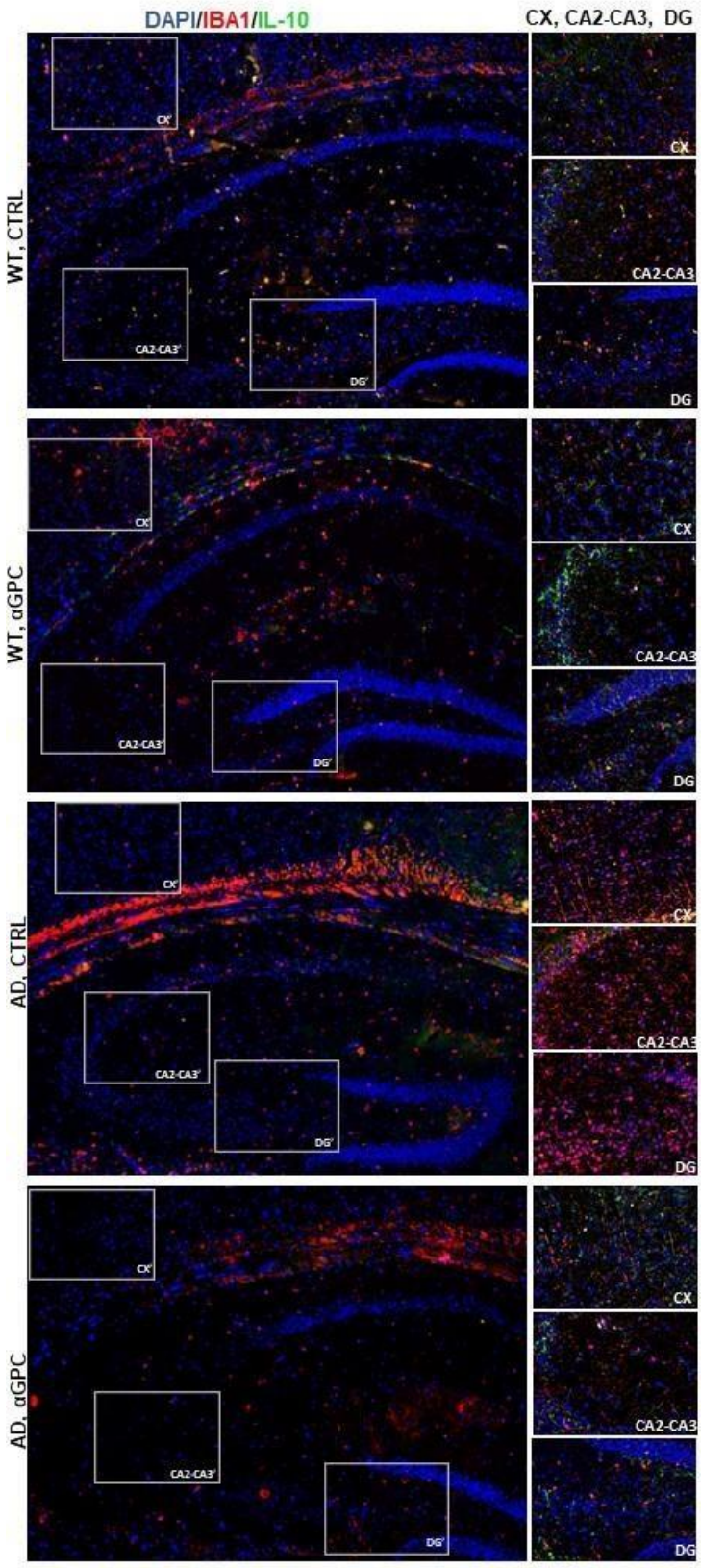

Figure 3: Anti-inflammatory cytokine IL-10 release is rescued by α-GPC treatment in 3xTg-AD mice.

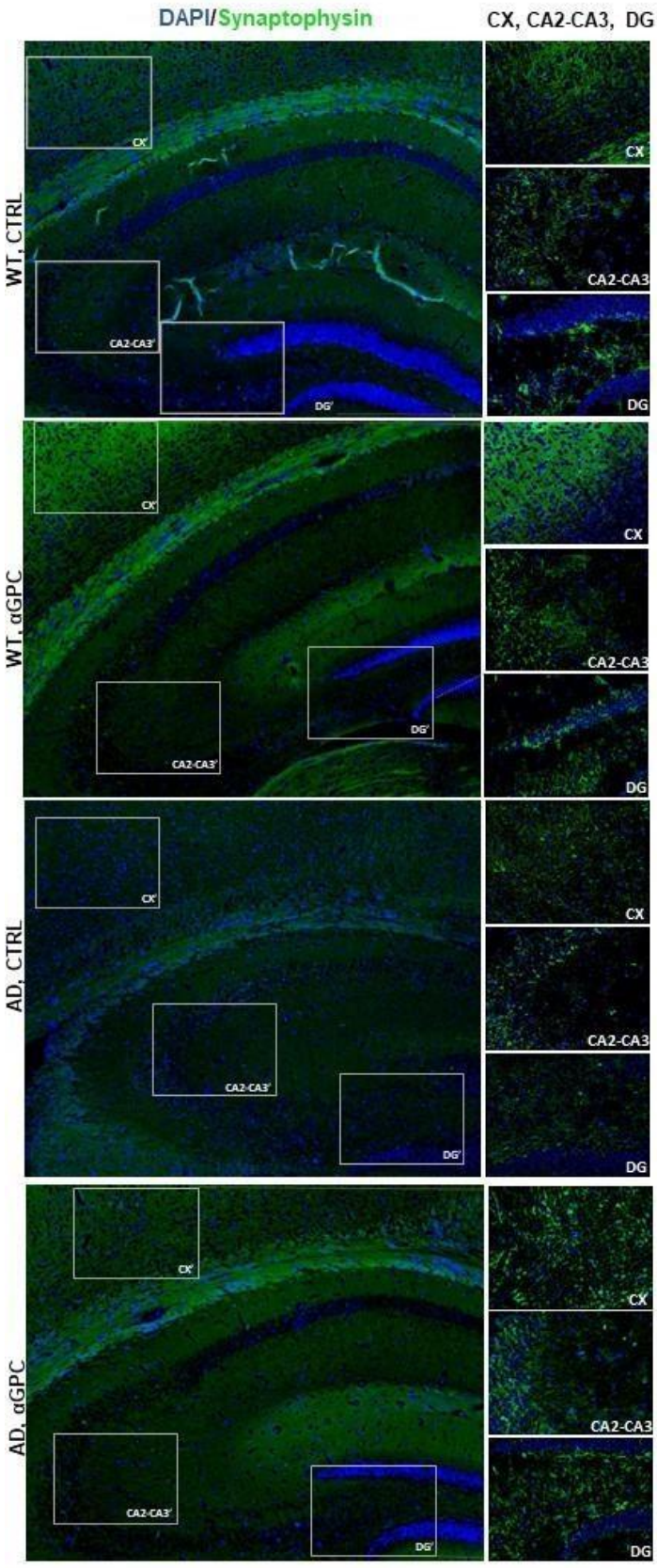

Figure 4: Effect of α-GPC treatment on neuronal plasticity in 3xTg-AD mice

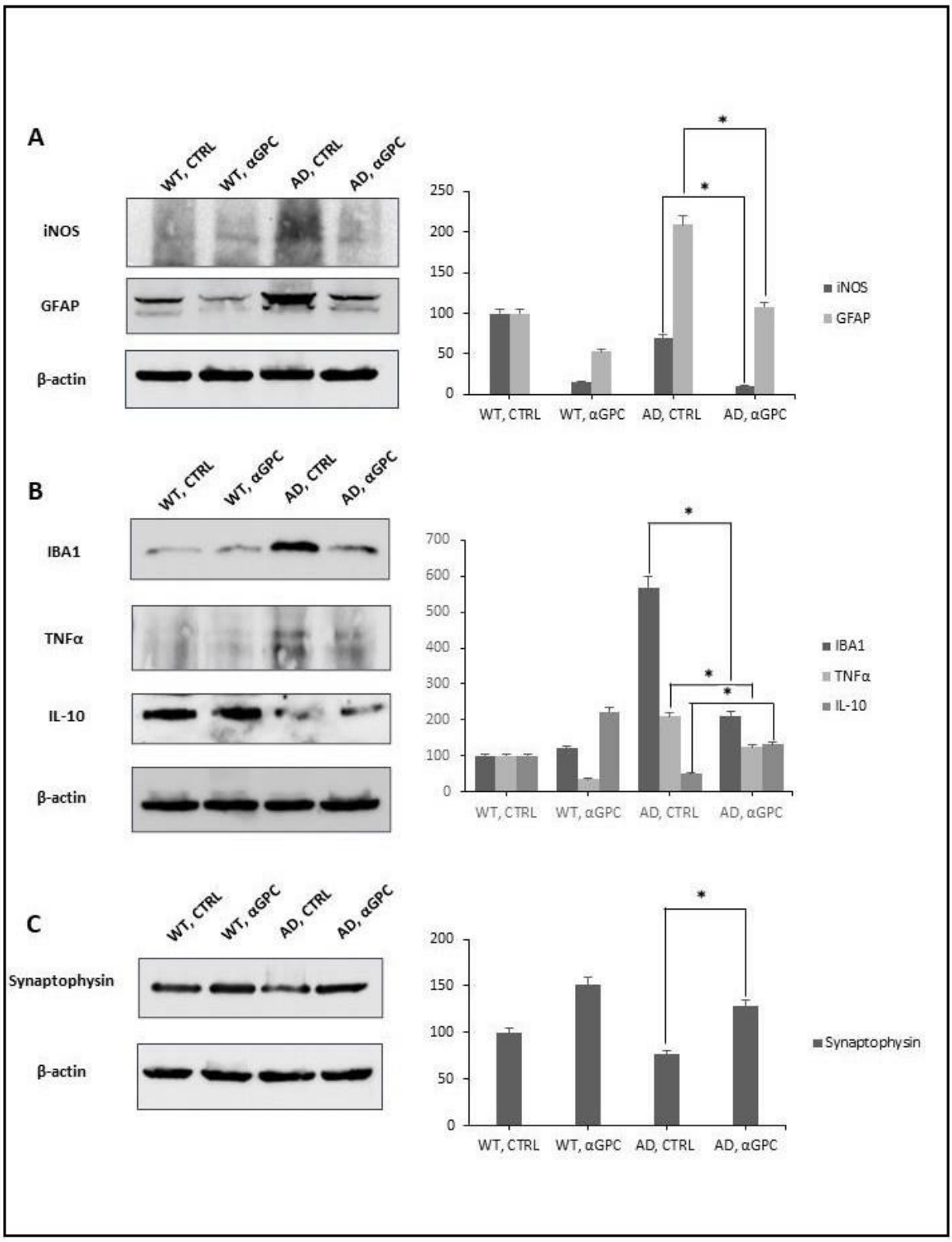

Figure 5: Western blot analysis of iNOS, GFAP (**A**), IBA1, TNF-α, IL-10 (**B**), Synaptophysin (**C**) in the hippocampus of WT and 3xTg-AD mice following or not chronic treatment with α-GPC and respective densitometric analysis.

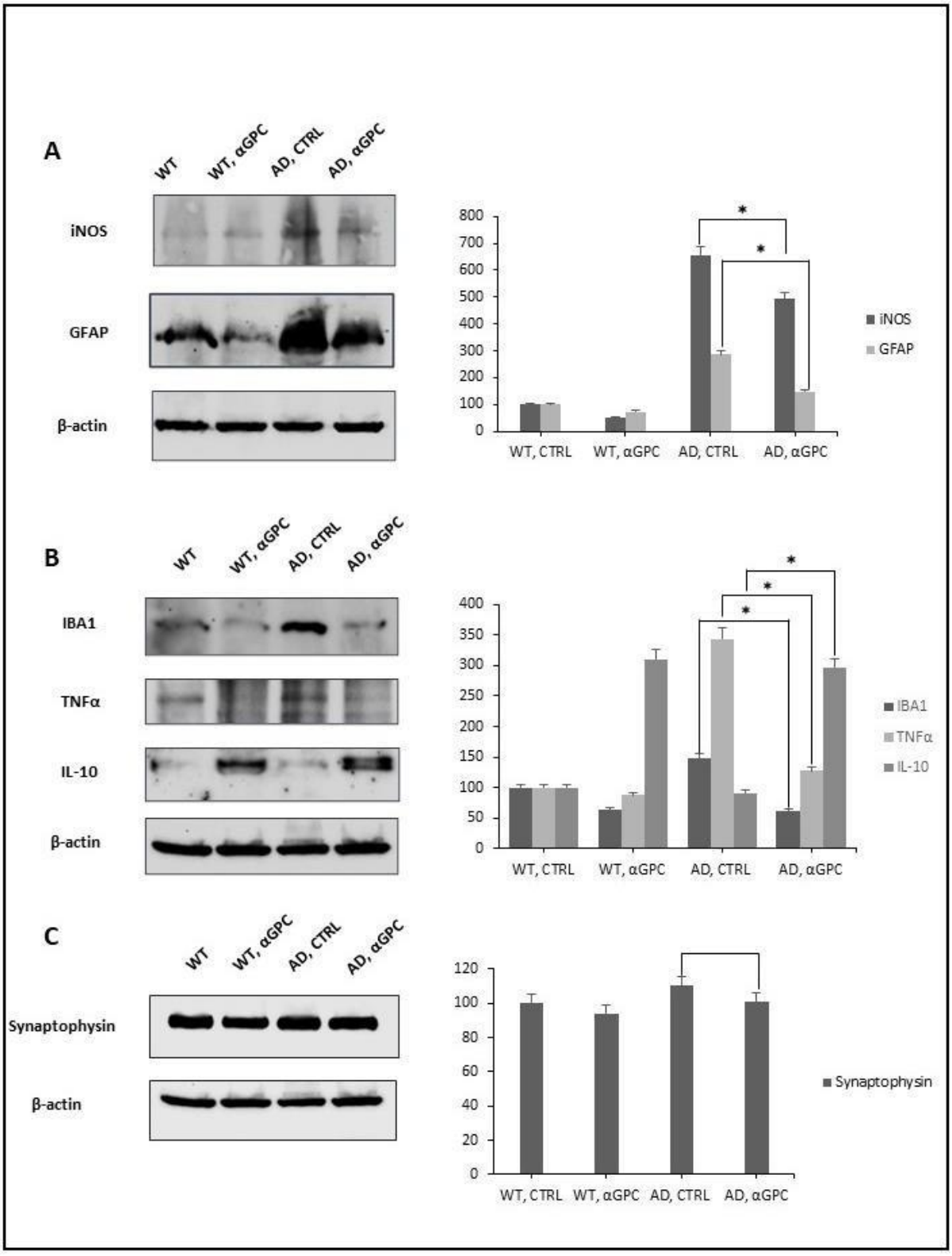

Figure 6: Western blot analysis of iNOS, GFAP (**A**), IBA1, TNF-α, IL-10 (**B**), Synaptophysin (**C**) in the cortex of WT and 3xTg-AD mice following or not chronic treatment with α-GPC and respective densitometric analysis.

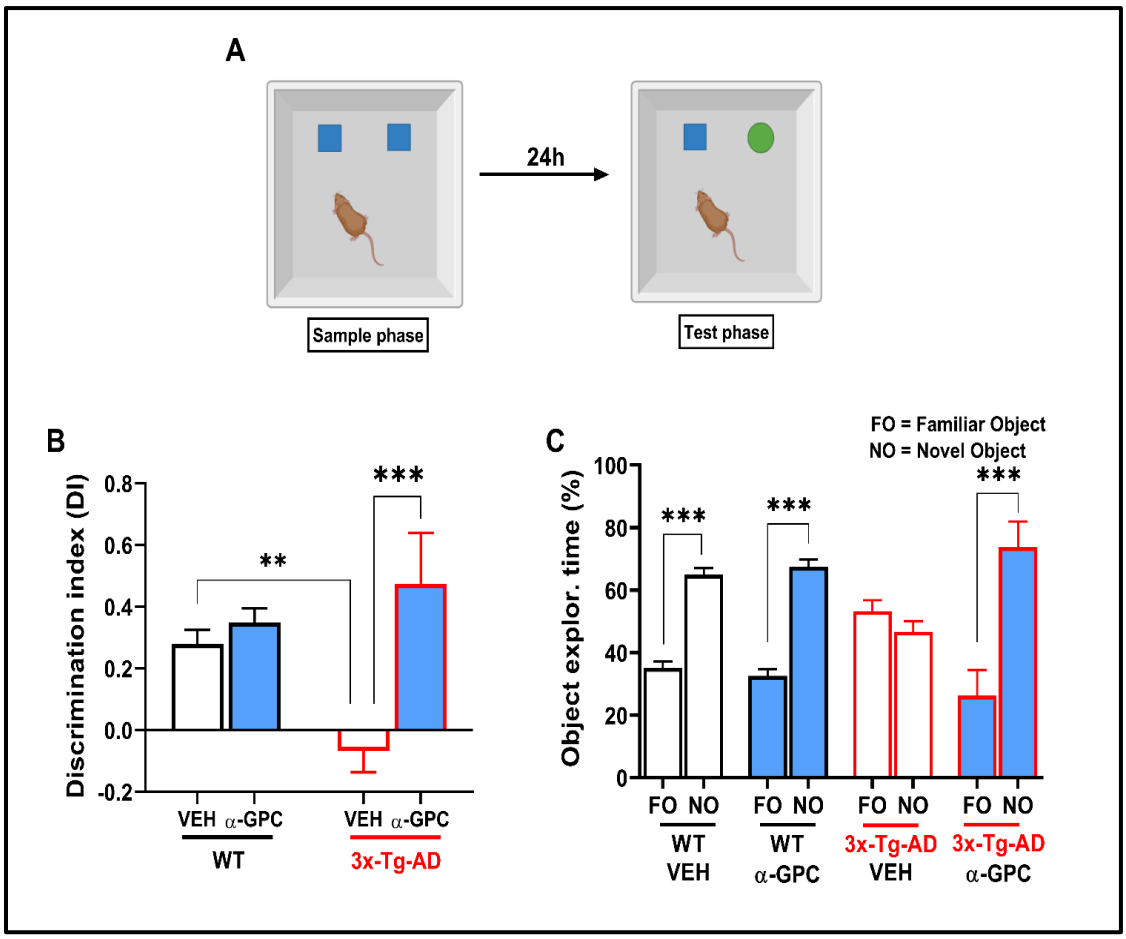

Figure 7: α-GPC rescued the long-term recognition memory deficits exhibited by 3xTg-AD mice in the NOR test.

(A) Experimental procedure conceived to evaluate the long-term (24-h delay) object recognition memory in WT and 3xTg-AD mice treated with α-GPC. WT treated with vehicle (N = 8); WT treated with α-GPC (N = 4); 3xTg-AD mice treated with vehicle (N = 4); 3xTg-AD mice treated with α-GPC (N = 3). **(B)** Discrimination index (DI) and **(C)** exploration time (%) of familiar object (FO) and novel object (NO) calculated to evaluate the cognitive performance of mice during the test phase of the NOR task. Two-way or three-way ANOVA followed by Bonferroni post hoc test: **p < 0.01 and ***p < 0.001. Values are expressed as means ± s.e.m.

DISCUSSION

Among choline precursors, α-GPC appears the most effective in enhancing in vivo Ach release, contributing to anabolic processes, and positively influencing membrane fluidity [5]. Several preclinical studies have demonstrated that α-GPC facilitates learning and memory, counteracting cognitive deficit in different experimental models of aging brain [32,33]. Moreover, α-GPC was proposed as potential neuroprotective agent for different pathological conditions based on inflammatory phenomena [34–36].

According to this evidence, the present study was designed to further investigate the long-term treatment effects of α-GPC in 3xTg-AD mice. This represents a well-established transgenic model displaying the attractive combination of both plaque and tangle development with the subsequent neurodegeneration, synaptic impairment, and cognitive deficits [14].

Alongside, neuroinflammation plays an important pathological role in AD brains, affecting cognition and memory [37]. This role is supported by epidemiological evidence that known risk factors for AD have a clear inflammatory component [38]. Additionally, the level of inflammation correlates with the severity of cognitive and non-cognitive symptoms of AD [39]. Inflammation often occurs in the hippocampus and cortex, as shown by an increased secretion of proinflammatory cytokines implicated in mediating neurodegeneration [40] Our results show a reduction of activated glia in the cortex and hippocampus of mice that received a chronic α-GPC treatment. In our hands, the immunofluorescence signal for GFAP and Iba-1 markers has been remarkably reduced in the brain of 3xTg-AD treated mice, further supporting the hypothesis that precursor-mediated enhancement of cholinergic transmission is able to reverse the sustained activation of glia fueling the neuroinflammatory machinery. Such increased expression of activated microglia and astrocytes is consistent with abundant expression and release of inflammatory mediators, such as iNOS, and TNF-α. Consistently, a positive effect of α-GPC treatment on hippocampus glial reaction was also documented in spontaneously hypertensive rats, a well-characterized model of vascular brain injury used to mimic some neuropathological changes occurring in vascular dementia [41].

Conversely, in basal conditions, namely in absence of a specific pro-inflammatory inducement, the administration of α-GPC seems not to be effective in modulating these pathways [42]. Probably, this compound presents an anti-inflammatory effect primarily in pathological conditions, most likely due to the increase in Ach levels. However, the

mechanism behind α-GPC supplementation's ability to inhibit these harmful features is not clear. Pharmacological studies have shown that choline and cholinergic precursors have anti-inflammatory effects in various pathological scenarios [43]. Such activity of choline may be identified as being due to stimulation of the alpha 7 nicotinic acetylcholine receptor (α7-nAChR) expressed by microglial cells [44,45], and its activation, inhibiting NLRP3 inflammasome, attenuates the proinflammatory response and prevents the production of reactive oxygen species [46].

The NOR test is a straightforward behavioral assay that rely primarily on the spontaneous behavior of rodents to explore novelty in the absence of externally applied reinforcement [47]. Several preclinical studies on transgenic models of AD have used this task to evaluate object recognition memory processes changes in hippocampal synaptic efficacy [48,49]. Actually, hippocampus represent an essential novelty detector due to its role in comparing previously stored information with new incoming aspects of one particular situation and the preference for a novel object means that presentation of the familiar object persists in animals' memory [50,51]. Based on the above evidence, we hypothesized that α-GPC, sustaining Ach release in the hippocampus, might counteract its relative functional decline. In order to support our hypothesis, we performed the NOR test to investigate the changes in cognitive function in 3xTg-AD mice and to evaluate the effect of chronic treatment with α-GPC. Our study results showed that mice treated with α-GPC significantly spent more time exploring the novel object while 3xTg-AD mice treated with vehicle spent approximately the same amount of time exploring both objects. This result has been corroborated by data obtained with Western blot analysis which showed that α-GPC treatment significantly reversed the down-regulated expression of hippocampal synaptophysin in 3xTg-AD mice.

In summary, our findings indicate that chronic treatment with α-GPC attenuates the progression of neurodegenerative damage, as well as by switching off neuroinflammatory features, which are known to be dysregulated in AD and in other neurodegenerative disorders, also sustaining the key function of hippocampal synapses in maintenance of a fairly regular cognitive status. Therefore, translationally, a therapeutic use of α-GPC could be thus envisioned in very early phases of AD, namely, for instance, during the appearance of first and subtle signs of cognitive decline.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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General discussion and conclusions

Although it has been more than 100 years since Alois Alzheimer first described the pathological signs associated with AD, still important gaps remain in our understanding of the condition and the nature of the pathological processes that underline the disease [6].

Although the etiopathogenesis of the disease is mainly based on accumulation of beta amyloid plaques and the neurofibrillary tangles of tau protein, the "amyloid cascade hypothesis" alone cannot fully explain the neuronal damage in AD. Neuroinflammation, in fact, plays a significant role in neurodegeneration related to AD.

Reiterating that AD is the most common form of dementia, characterized by cerebral atrophy and cognitive decline, important critical contributors of the neuroinflammation response are the chronic activation of the immune cells resident in the brain and the release of inflammatory and neurotoxic factors.

Since decades, in fact, active research has investigated network connections between the immune and the nervous systems: this reciprocal functional control is an essential mechanism to tissue repair and regeneration as well as removal of damaged tissues and cells.

Although inflammation is intended to be protective, an excessive inflammatory response can cause or contribute to tissue damage. Neuroinflammation is to be considered as an inflammatory response in the CNS secondary to a neuronal insult and it configure as a vicious cycle of activation of glial cells that trigger the release of proinflammatory factors and neuronal damage.

In relation to the nature of the signal, microglia orchestrate a specific response, through different receptors and signaling pathways, which includes phagocytosis, increased migration, proliferation, and release of bioactive molecules.

In this scenario, interventions to target crucial immune pathways in the pre-disease period and to modulate the immune response along the disease process could bring about promising outcomes as part of a disease-modifying therapeutic strategy.

Consistently, in this work we focused our interest on the molecule of α-GPC, significantly effective in enhancing Ach synthesis and release, probably due to its ability to cross the BBB and contribute to nerve cell/synaptic membrane

reorganization.
Considering the protective action of α-GPC on cholinergic neurotransmission, the first aim of our study has been to investigate its effects on Aβ-induced inflammation in an *in vitro* model of microglia; moreover, we set the goal of evaluating the neuroprotective effects of the molecule in a mouse model of AD (3xTg-AD).

As reported in **Chapter I**, the first goal of the project has been testing the effect of α-GPC as an effective agent for enhancing cholinergic transmission. We studied its impact on microglial phenotypic switching using an *in vitro* model of BV2 culture cells treated with Aβ, examining the involvement of α7 nAChR in this effect. We first assessed the impact of α-GPC on BV2 cells viability and its potential protective role against Aβ-related toxicity in the same cell line. We showed that at different concentrations, α-GPC did not interfere with cell proliferation and/or survival, while it effectively mitigates Aβ-induced detrimental effect in BV2 microglial cells at all the time point studied.
Our findings demonstrate that α-GPC has the ability to induce a switch in microglial phenotype from the proinflammatory M1 to the anti-inflammatory M2 status mediated by nAChR α7.
Overall, our results suggest that α-GPC may have an anti-inflammatory role in AD, possibly exerting its beneficial effects by enhancing cholinergic activity that could antagonise microglial-mediated inflammation, activating the α7 nAChR system. In view of the above, it is plausible that therapeutic use of α-GPC may be a promising strategy to reduce AD-related neuroinflammation.

The second part of the study reported in **Chapter II** was aimed to further investigate the long-term treatment effects of α-GPC in 3xTg-AD mice.

Our results showed a reduction of activated glia in the cortex and hippocampus of mice that received a chronic α-GPC treatment. In our hands, the immunofluorescence signal for GFAP and iNOS markers of reactive astrocytes and for Iba-1 and TNF-α proinflammatory microglia markers has been remarkably reduced in the brain of 3xTg-AD treated mice. On the other hand, α-GPC increases the expression of anti-inflammatory IL-10 expressing microglia and of the synaptic marker synaptophysin in the hippocampus. These results further support the

hypothesis that precursor-mediated enhancement of cholinergic transmission is able to reverse the sustained activation of glia that fuel the neuroinflammatory machinery in AD.

Furthermore, we observed that α-GPC was effective in restoring cognitive behavior as demonstrated by the novel object recognition test.

In summary, our findings indicate that chronic treatment with α-GPC attenuates the progression of neurodegenerative damage, either by contributing to phenotypic switching of brain resident innate immune cells, astrocytes and microglia, as well as by sustaining the key function of hippocampal synapses in the maintenance of a regular cognitive status. Therefore, therapeutic use of α-GPC could be thus envisioned as a promising therapeutic approach during the appearance of first disturbances concerning minimal cognitive impairment (MCI). Although AD in the latter stage is not yet in its overt phase, it could be advantageous to treat patients with an agent which is not invasive, but that might warrant keeping neuroprotective mechanisms active.

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Statements

- *Neuroinflammation and oxidative stress play a key role in the onset and course of neurodegenerative and demyelinating diseases and in psychiatric and neurodevelopmental disorders.*
- *The determining meaning of neuroinflammation consists in the activation of microglial cells and the accumulation of amyloid plaques and tau protein around neurons.*
- *Cholinergic function modifications are implicated in the pathogenesis of the brain changes that occur in many forms of dementia, including Alzheimer's disease; therefore, cholinergic precursors are suitable to treat the cholinergic dysfunction and the subsequent cognitive decline.*
- *Looking at things from another perspective can suggest new points of view: although α-GPC is a molecule already widely known and studied, it seems capable of supporting new scientific evidence.*
- *As the prevalence of Alzheimer's increases, so does the urgency to find a cure.*

- *"I am grateful to all those people who told me NO. It's thanks to them that I am what I am" – Albert Einstein.*
- *"Whatever you can do, whatever dream you can dream, begin. Audacity brings with it genius, magic and strength. Begin now" – Goethe.*
- *"TRAIN while others sleep, STUDY while others quit, RESIST while others give up, at the end you will live what others dream of" – Anonymous.*
- *"Don't shorten your morning by getting up late. Think of it as the quintessence of life, somehow sacred" – Arthur Schopenhauer.*
- *"If it's not right don't do it, if it's not true don't say it" – Marco Aurelio.*