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TNFα increases STAT3-mediated expression of glutaminase isoform KGA in cultured rat astrocytes

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ABSTRACT

Glutamate related excitotoxicity and excess of cerebral levels of tumor necrosis factor alpha (TNFα) are interrelated and well documented abnormalities noticed in many central nervous system diseases. Contribution of kidney type glutaminase (KGA) and shorter alternative splicing form (GAC) to glutamine degradation in astrocytes has been recently a matter of dispute and extensive study but the regulation of the GLS isoforms by inflammatory factors is still not well known. Here we show that treatment of cultured rat cortical astrocytes with pathophysiologically relevant (50 ng/ml) concentration of TNFα specifically increases the expression of KGA but not GAC and increases activity of GLS. No changes in the expression of either of two GLS isoforms were observed following treatment with other tested cytokines IL-1β and IL-6. The TNFα mediated KGA expression was associated with increased phosphorylation of signal transducer and activator of transcription 3 (STAT3). Stimulatory effect of TNF-α on KGA expression was reduced by selective inhibition of (STAT3) but not by inhibition of STAT1 nor nuclear transcription factor kappa. Additionally, the role of miRNA in TNFα–induced expression of KGA in astrocytes was excluded, since the expression of miR-23a/b and miR-200c, potential regulators of KGA expression, was unchanged. This study documents increased KGA expression in the astrocytes under inflammatory stimulation, identifying TNFα as a cytokine mediating this response, and demonstrates the specific and selective involvement of STAT3.

1. Introduction

The majority of pathological conditions of the CNS are associated with intense inflammatory response and excitotoxicity induced by excessive extracellular cerebral glutamate (Glu). These include diseases associated with physical tissue damage such as stroke [1] and traumatic brain injury [2], as well as neurodegenerative disorders: Alzheimer's, Parkinson's and Huntington's diseases [3] and also become a part of systemic inflammatory responses associated with sepsis [4]. The essential event in the inflammatory response is an excess of cerebral level of "proinflammatory" cytokines including tumor necrosis factor alpha (TNFα) and interleukins (IL) like IL-1 and IL-6. While these small soluble proteins are critical factors in the regulation of cellular immunity their function is remarkably pleiotropic [5]. The inflammatory response has long been considered to be a domain of microglia, however more recent evidence implicates astrocytic contribution as well [4], despite the frequently envisaged function of astrocytes in the maintenance of the Glu and glutamine (Gln) homeostasis in CNS offering the protection to neurons from excitotoxicity. One of the key reaction in Glu/Gln metabolism is the hydrolytic deamidation of Gln to Glu and ammonium ions catalyzed by mitochondrial enzyme glutaminase (GLS). The fact that in different pathological conditions brain excitotoxicity is coupled to inflammatory response, prompted an idea that the two roles of astrocytes: contribution in neuroinflammatory response and excitotoxicity may be mechanistically linked.

In mammals two analogous genes encode distinct GLS isozymes: *gls1* encodes kidney type (KGA) isoform, while *gls2* gene codes liver-type (LGA) isoform [6]. The classical pattern of GLS expression has been recently challenged by the discovery of novel transcript variants and protein isoforms. Besides KGA, *gls1* encodes a shorter alternative splicing form with unique COOH terminal protein sequence called GAC [7]. This two isoformes can be considered together as GLS1. Recent findings

Abbreviations: CNS, central nervous system; GAC, glutaminase C isoform (protein coded by gls1); Gln, glutamine; GLS, glutaminase; Glu, glutamate; HPLC, High-performance liquid chromatography; IL, interleukin; KGA, kidney type glutaminase isoform (protein coded by gls1); LGA, liver type glutaminase (protein coded by gls2); NFkB, nuclear factor kappa B; PBS, phosphate buffer saline; TNFα, tumor necrosis factor alpha

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indicate that in neurons and microglia TNFα frequently named as a master cytokine contributing to neuroinflammatory cascade, elicits the inflammatory response by a mechanism involving activation of Gln degradation by specific activation of GLS1 [8,9].

A very recent report implicated GAC as a target of TNFα in the inflammatory response of astrocytes [10]. However, the same group [8] demonstrated that in neurons, KGA but not GAC is specifically stimulated by TNFα. The aim of our study was to evaluate whether and *via* what mechanism two GLS isoforms, KGA and GAC, respond to TNFα or other proinflammatory cytokines in astrocytes. To answer these questions, rat cortical astrocytes in culture were treated for 48 h with different, relevant to those observed in CNS pathological states, concentrations of TNFα, IL-1 and IL-6 and the expression of GLS1 isoforms were assessed. We established that TNFα was an only effective stimulating agent and KGA the only responsive GLS1 isoform, than next we focused our investigation on the underlying mechanism of the TNFα-KGA direct relation.

2. Materials and methods

2.1. Cell culture and treatments

All studies were carried out according to, EU Directive 2010/63/EU for animal experiments and protocols were approved by the 2nd Local Ethical Committee (Warsaw, Poland; permission No. 54/2015). Primary astrocyte cultures were prepared from cortices of newborn Wistar rats [11]. Briefly, cerebral cortex was isolated on ice, then passed through Nitex nylon netting (pore size 80 μm) into Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies) containing 20% fetal bovine serum; Gibco, Life Technologies). Cells were grown in 37 °C in humidified atmosphere of 95% air and 5% CO_2. Experiments were performed on 3-week astrocytes: non treated (control) or treated for 48 h with: (i) 10; 50 ng/ml TNFα (ii) 5; 10 ng/ml IL-1β; (iii) 5; 10 ng/ml IL-6 (Sigma-Aldrich; St. Louis, MO, USA). The nuclear factor kappa β (NFκβ) inhibitor - Bay-11-7082 (10 μM, Sigma-Aldrich; St. Louis, MO, USA), STAT-1 inhibitor - fludarabine phosphate (50 μM, Sigma-Aldrich; St. Louis, MO, USA) and STAT-3 inhibitor STA-21 (20 μM, Santa Cruz Biotechnologies, Dallas, TX, USA) were added to cell culture medium 30 min before TNFα. The activity of GLS was inhibited using 6-Diazo-5-oxo-L-norleucine (DON, GLS inhibitor) or BPTES (GLS1 specific inhibitor; Sigma-Aldrich; St. Louis).

2.2. RNA isolation and PCR reaction

Total RNA from cultured astrocytes was extracted by Tri-Reagent (Sigma-Aldrich; St. Louis, MO, USA). 1 ng of RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA, USA). The rat *kga* and *gac* transcripts were amplified using specific primers synthesized by Institute of Biochemistry and Biophysics, Warsaw, Poland. Expression of rat β–actin was used as an internal control. The cycling conditions included an initial denaturation step at 94 °C for 5 min, followed by 45 cycles of 30 s at 94 °C, 1 min at 62 °C and 1 min at 72 °C, and a final extension step of 10 min at 72 °C. 5 μl of the PCR products were run on a 2% agarose gel and visualized using ethidium bromide dye. The relative level of microRNA was evaluated by real-time PCR analysis using Applied Biosystems Taqman probe assays (miR-23a assay ID 000399, miR23-b assay ID 000400, miR-200c assay ID 463287_mat) and U6 snRNA(assay ID 001973) as endogenous control. The reaction and data collection were performed on ABI 7500 apparatus (Applied Biosystems). The results of the analysis were presented according to an equation ($2^{-\Delta\Delta Ct}$) that gives the amount of target, normalized to an endogenous reference, and relative to a calibrator. Ct is the threshold cycle for target amplification [12].

2.3. Protein extraction and Western blot analyses

Astrocytes were washed in PBS and homogenized by sonication in RIPA Lysis and Extraction buffer (Sigma-Aldrich; St. Louis, MO, USA) containing Protease Inhibitor Cocktail (concentration 1:200, Sigma-Aldrich; St. Louis, MO, USA) phosphatase inhibitor cocktail (concentration 1:100, Sigma-Aldrich; St. Louis, MO, USA) and 5 mM sodium fluoride (Fluka, Sigma-Aldrich) and then centrifuged for 10 min at 12000 × g and 4 °C. Equal amounts of protein (30 μg) were separated on 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in TBS-Tween buffer (50 mM Tris; 150 mM NaCl; 0.1% Tween 20). The membranes were than incubated over-night with anti-KGA antibody (1:1000 Cell Signaling, Danvers, MO, USA) and anti-GAC (1:250 ProteinTech, Manchester, UK), followed by 1 h incubation with HRP-conjugated-secondary IgG antibodies (1:5000, Sigma-Aldrich; St. Louis, MO, USA) for detection by Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The first antibody was stripped off with 0.1 M glycine, pH 2.9, and second incubation was performed with an antibody against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 1 h incubation at 20–22 °C (1:7500, HRP-60004, ProteinTech, Manchester, UK). The chemiluminescent signal acquisition and densitometry analysis were conducted using the G-Box system (SynGene,) and GeneTools software (SynGene, Bengaluru, India). Total protein concentration was determined by the Lowry method using Modified Lowry Protein Assay Reagent (Pierce, Thermo Fisher, Waltham, MA, USA).

2.4. GLS activity assay

GLS activity measurement was proceed by the method described by Romero-Gomez with modifications [13]. Briefly, 20 μl of astrocytic cell lysate prepared as described were mixed with 100 μl of reaction medium containing: 150 mM K_2HPO_4, 171 mM L-glutamine at pH 8.6. Blanks were prepared as followed with addition of 10 μl of 10% trichloroacetic acid (TCA). After 60 min incubation in water bath (37 °C), reaction was stopped with 10% TCA. The samples were placed on ice for 15 min and centrifuged at 12,000 RPMI. Supernatant were then loaded into micro-titrate plate and OPA based reagent (0.2 M K_2HPO_4; 56 ml/L ethanol; 10 mM O-phthaldialdehyd; 0.5 mM β-mercaptoethanol) was added. Plate were kept in dark for 45 min at room temperature. Absorbance was measured at 405 nm with spectrophotometer (Bio-Rad-680, Bio-Rad Hercules, CA, USA). Standard curve of NH_4Cl (50–500 mg/L) in reaction medium was used for the calculation of the results.

2.5. Measurement of Glu/Gln concentration

The Glu and Gln concentration were analyzed in astrocytes culture lysates using High-performance liquid chromatography (HPLC) with fluorescence detector RF 2000 (Dionex, Thermo Fisher, Waltham, MA, USA) after derivatization in a timed reaction with O-phthaldialdehyd, as described earlier [14]. Samples were injected onto 150 × 4.6 mm 5 μm Hypersil Gold column, eluted with a mobile phase of 0.075 M KH_2PO_4 solution containing 10% (v/v) methanol, pH 6.2 (solvent A), and methanol (solvent B). The methanol gradient was 20–70% and the elution time was 30 min (1.2 ml/min). The separated amino acids were detected with fluorescence and amounts calculated using a standard curve derived from standard solutions of amino acids.

2.6. Confocal microscopy

Astrocytes cultured on glass microscopic slides placed in 24-well plates, were washed with PBS and fixed using 4% PFA for15 min. After washing with PBS, the samples were permeabilized for 15 min with 0.1% Triton X-100 and blocked for 1 h using 10% normal goat serum at

room temperature. The anti-KGA antibody was applied overnight at 4 °C (1:500 Cell Signaling, Danvers, MO, USA), The secondary antibody goat anti rabbit IgG H + L (Alexa 546, Invitrogen) was applied for 1 h at room temperature. Cell nuclei were contra-stained with DAPI (Dako, Agilent Technologies, Santa Clara, CA, USA) for 15 min. Mitochondria were stained with 200 μM Mito Tracker (Thermo Fisher, Waltham, MA, USA). After washing with PBS the samples were closed with Fluorescent mounting medium (Dako, Agilent Technologies, Santa Clara, CA, USA) and visualized using an Axio Observer Z.1 microscope confocal LSM 780 system (Carl Zeiss GmBH, Jena, Germany).

2.7. Statistical analysis

Obtained data were analyzed by one-way ANOVA with a post hoc Dunnet's test. All analyses were performed using 5.0 Graph Pad software (San Diego, California, USA). Values were expressed as mean ± S.D; $p < 0.05$ or less was considered statistically significant.

3. Results

3.1. The effect of TNFα, IL-1, IL-6 on KGA expression in astrocytes

Treatment with 50 ng/ml TNFα, a dose observed in pathophysiological conditions and used by others in an *in vitro* studies [10,15,16], significantly increased the expression of *kga* by ~50%. Neither 10 ng/ml TNFα nor other tested cytokines IL-1β and IL-6 did not affect *gls1* expression (Fig. 1). The KGA protein level was increased by ~40% only in 50 ng/ml TNF-α treated astrocytes. Unlike KGA the GAC coding mRNA and GAC protein level was unchanged by other cytokines

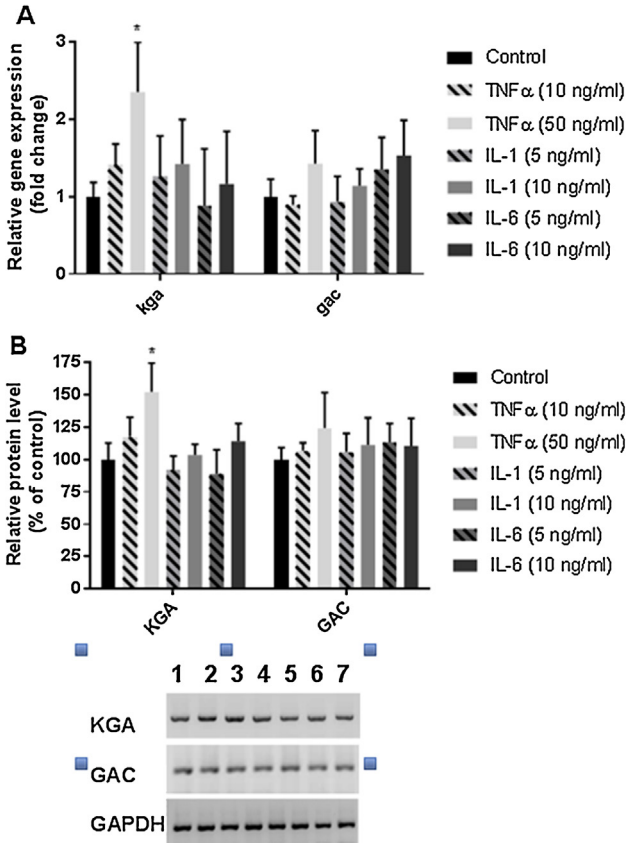

Fig. 1. Effect of cytokines on *gls1* expression (panel A) and KGA and GAC protein level (panel B) in rat cortical astrocytes treated for 48 h with: (i) 10; 50 ng/ml TNFα (ii) 5; 10 ng/ml IL-1β (iii) 5; 10 ng/ml IL-6. Results are mean ± SD (n = 6); *$p < 0.05$ vs control. Below representative picture of WB membrane. Numerated rows represent the same order as graphs.

treatment (Fig. 1).

The stimulatory effect of 50 ng/ml TNF-α on KGA protein level was also observed using confocal microscopy. The intensity of fluorescence after KGA staining was higher in images of TNFα treated astrocytes. It is worth noticing that fluorescence of KGA directly overlaps mitochondrial marker Mito Tracker Red (Fig. 2).

3.2. The activity of GLS and Glu/Gln concentration in TNFα-treated astrocytes

The total GLS activity in astrocytic cell lysate was increased by ~40% after TNFα incubation. Pre-treatment with GLS inhibitor DON strongly decreased (~50%) GLS activity and attenuated the effect of TNFα (Fig. 3). The analysis was supported by results obtained from the measurement of intracellular Glu and Gln concentration (Fig. 4). The content of Glu was elevated in TNFα treated cells and attenuated by using more specific GLS1 inhibitor BPTES. In addition, the rise in Gln concentration by ~30% was observed after BPTES treatment in astrocytes incubated with TNFα (Fig. 4).

3.3. The miR- 23a/b and miR- 200c expression level is not changed in TNFα-treated astrocytes

Taking into account that recently published studies demonstrated that miR-23a/b [17] and miR-200c [18] regulate *gls* expression, we analyzed the expression profile of selected miRNAs. Unchanged miR-23a/b and miR-200c expression (Fig. 5) indicates that those miRNAs are not crucial in TNFα –induced regulation of KGA expression in cultured astrocytes.

3.4. STAT3 activation is necessary to induce KGA expression by TNFα

Further we examine a possible role of three transcription factors NF*k*B, STAT1 and STAT3 in TNFα-induced up-regulated expression of KGA using selective inhibitors. Treatment of astrocytes with STAT3 inhibitor (STA21) largely reduced the stimulatory effect of TNFα on KGA (Fig. 6), whereas the other compounds were not effective. Additionally we documented TNFα-induced STAT3 phosphorylation in astrocytes in the time course manner (Fig. 7). The highest level of p-STAT3 was noticed after 24 h of TNFα stimuli which preceded the induction of KGA expression (time course of KGA expression not shown).

4. Discussion

It has been documented that glutaminase (GLS), the enzyme degrading Gln to Glu, is ubiquitously present in all the cell types of the CNS: neurons [19], astrocytes [20] and microglia [21]. However, studies on the role of glutamatergic neurotransmission have been focused on its neuronal aspect, where neuronal GLS has been considered as critical for proper operation of Glu/Gln cycle, in which astrocytes and neurons cooperate in the synthesis of neurotransmitters: Glu and GABA [22,23]. Therefore knowledge on the isoforms representation and function of GLS located in astrocytes has considerably lagged behind that of their neuronal counterparts. While the predominant expression of both *gls1* isoforms in contrast to *gls2* products has been documented in cultured astrocytes [24] and later confirmed in human autopsy and rodent brain tissue [25], their specific function has remained obscure. Only a few studies have drawn attention to the role of Gln degradation by GLS in inflammatory response. Previous works showed that TNFα regulates the expression of GLS in microglia and neurons [8,9]. Interestingly, in cultured neurons TNFα (and also IL-1β) stimulated the expression of the KGA, but GAC protein [8]. This led to formulation the hypothesis implicating TNFα as a key player and potential therapeutic target in neurodegenerative diseases [26]. Evidence is accumulating that apart from microglia, astrocytes are an important source of cytokines as well, either acting in an autocrine manner or affecting adjacent

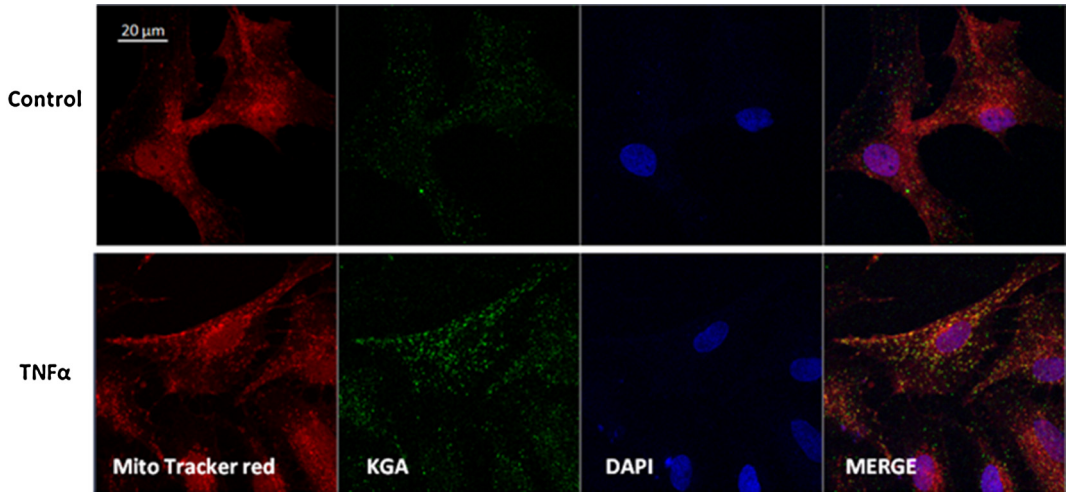

Fig. 2. 1 Immunofluorescence labeling of (i) KGA (green), (ii) mitochondria with Mito Tracker Red (red) (iii) cell nuclei DAPI staining (blue) in astrocytes treated for 48 h with 50 ng/ml TNFα. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

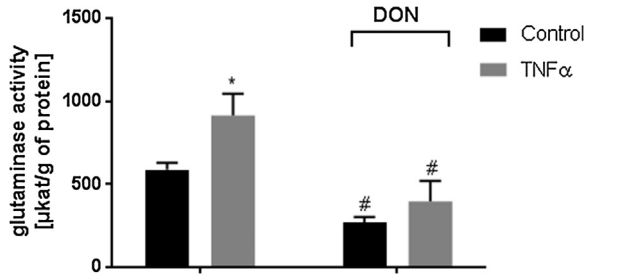

Fig. 3. Effect of 48 h incubation with 50 ng/ml TNFα on GLS activity. Results are mean ± SD (n = 4); *p < 0.05 vs control, #p < 0.05 vs corresponding DON non-treated group.

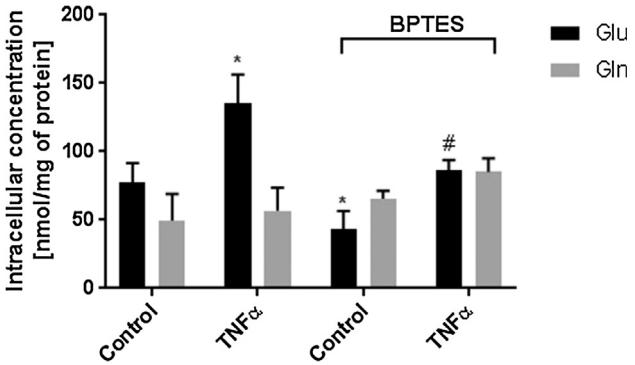

Fig. 4. Intracellular concentration of Glu and Gln in rat cortical astrocytes treated with 50 ng/ml TNFα Results are mean ± SD (n = 6); *p < 0.05 vs control, #p < 0.05 vs TNFα.

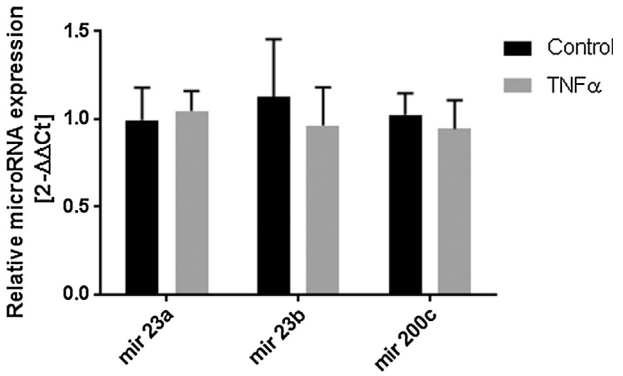

Fig. 5. The relative level of miR 23a/b and miR-200c in rat cortical astrocytes treated with 50 ng/ml TNFα. Results are mean ± SD (n = 3).

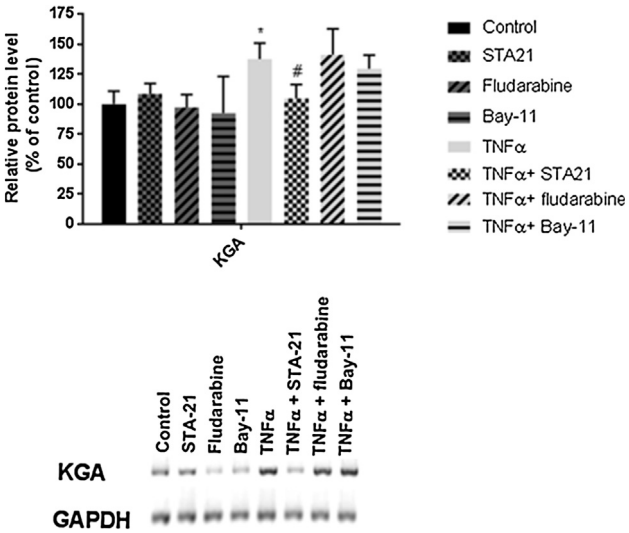

Fig. 6. The protein level of KGA after TNFα treatment and pharmacological inhibition of transcription factors STAT 1 (fludarabine), STAT3 (STA21) and NFkβ (Bay-11). Results are mean ± SD (n = 4); *p < 0.05 vs control, #p < 0.05 vs TNFα. Below representative picture of WB membrane.

brain cells [27,28]. Novelty of the present study is that TNFα affects KGA, the other GLS isoform, extending a list of TNFα targets. Of note in this context, KGA was earlier reported to be responsive to this cytokine in neurons [8]. The discrepancies between the results of Wang et al. (2017) described in the Introduction and the present study may lay in the methodology used (species differences of derived cells and/or in particular elements of the experimental procedure). The study of Wang et al. (2017) was carried out on mouse astrocytes (C57BL/6J mice) whereas the present study employed astrocytes from rat (Wistar strain). It is well established that primary cultures of rat and mice astrocytes present different morphology and show different expression pattern of key astrocytic proteins such as GFAP and vimentin [29]. Specifically, rat astrocytes cultured *in vitro*, express significantly more GFAP and vimentin of different molecular size that mouse astrocytes, altogether presenting more proliferative phenotype than mouse astrocytes [30]. The species specific response of astrocytes to neuroinflammation was

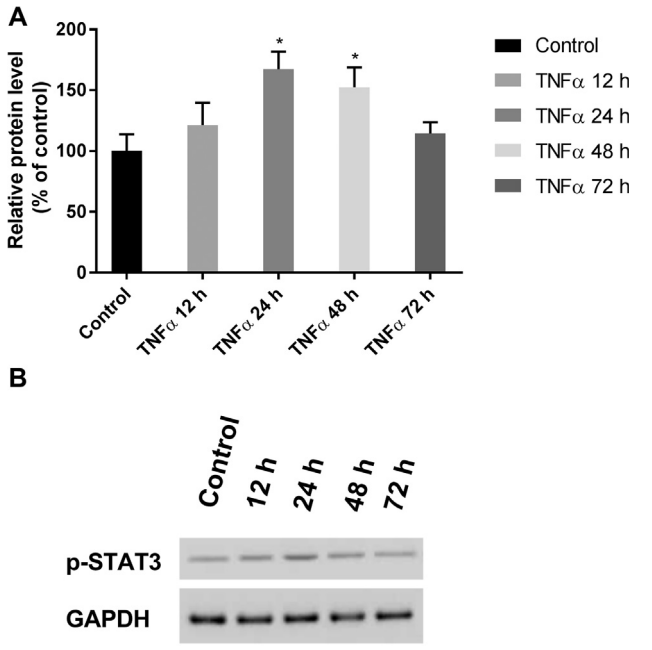

Fig. 7. Panel A. The STAT3 phosphorylation in rat cortical astrocytes after various time of TNFα treatment. Results are mean ± SD (n = 6); *p < 0.05 vs control, #p < 0.05 vs TNFα. Panel B representative picture of WB membrane.

also demonstrated in a comparative *in vivo* study [31]. One other, albeit presumably less critical difference between the procedures used in the study of Wang et al. (2017) and the present study was TNFα exposure duration (24 h vs 48 h). Nevertheless, it is worth emphasizing that our manuscript and that of Wang et al. (2017) agreeably point to the enhancement of GLS1 activity as so far not investigated in detail effect of TNFα action in astrocytes, and the increase of *gls1* expression as the underlying mechanism of this phenomenon.

Most importantly, the present study delineated selected aspects of the molecular mechanism involved in the stimulatory effect of TNFα on GLS1 expression and activity. The conceptual framework of the mechanistic hypotheses and compatibility of the present results with the hypotheses deserve a comment.

A recent work showed that in P-493B lymphoma cells and PC3 prostate cancer cells, *gls1* expression is positively regulated by miRNA (miR-23a/b) [32]. In human leukemic Jurkat cells the NF-κB p65 subunit inhibited miR-23a expression which resulted in enhanced GLS expression [33]. Bearing in mind that TNFα is a well-known stimulator of NF-κB pathway we hypothesized that miR-23a/b may be involved in TNFα induced *gls1* activation in astrocytes. Likewise recently published study obtained on rat heart revealed that miR-200c binds to mRNA coding GLS suppressing GLS expression [18]. However, the present data failed to confirm our hypothesis: the level of miR-23a/b and miR-200c was not affected by TNFα treatment.

In the next step, using pharmacological inhibitors, we tested directly the role of NF-κB. We also examined the potential contribution of STAT1, which was previously implicated as a stimulant of GLS1 expression in HIV-1 infected macrophages [34], and STAT3. The present study does not bespeak the role of STAT1. However, the novel finding of this study is the involvement of STAT3 as a mediator of KGA overexpression by TNFα in astrocytes. This inference was additionally confirmed by demonstration of increased STAT3 phosphorylation in TNFα-treated astrocytes. The JAK/STAT3 pathway is known to trigger astrocyte reactivity in models of acute injury [35] and in Alzheimer's and Huntington's diseases [36]. A potential link between STAT3 and Gln/Glu metabolism came to light in a recent study demonstrating activation of STAT3 by Gln in cancer cells [37]. It remains to be examine whether regulation of STAT3 by Gln pertains to the inflammatory response in non-malignant CNS cells.

It's important to underlie that TNF can cause Glu accumulation not only by increasing Glu production by enhancing GLS activity and/or its expression, but also by simultaneous inhibition of Glu reuptake towards reducing Glu clearance. Of note in this context, accumulated evidence related to TNF-induced excitotoxicity through the inhibition of Glu reuptake in different CNS pathologies were provided [38,39]. This aspect however is beyond the scope of present work and deserve a detailed study.

Summarizing, this study underscores the role of astrocytes in the inflammatory response observed in different CNS diseases associated with increased TNFα, revealing that KGA may serve as mediator of excitotoxicity-promoting action during inflammation. Clearly, to account for a specific role of the interaction between different CNS cell types in the manifestation of the inflammatory response, the evidence presented in this study requires verification in the *in vivo* setting.

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Declaration of Competing Interest

The authors of the article untitled "TNFα increases STAT3-mediated expression of glutaminase isoform KGA in cultured rat astrocytes" declare that they have no competing interests.

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