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Inflammatory Cytokine, IL-1β, Regulates Glial Glutamate Transporter via microRNA-181a *in vitro*

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Abstract. Glutamate overload triggers synaptic and neuronal loss that potentially contributes to neurodegenerative diseases including Alzheimer's disease (AD). Glutamate clearance and regulation at synaptic clefts is primarily mediated by glial glutamate transporter 1 (GLT-1). We determined that inflammatory cytokines significantly upregulated GLT-1 through microRNA-181a-mediated post-transcriptional modifications. Unveiling the key underlying mechanisms modulating GLT-1 helps better understand its physiological and pathological interactions with cytokines. Primary murine astrocyte and neuron co-culture received 20 ng/mL IL-1β, TNF-α, or IL-6 for 48 h. Soluble proteins or total RNA were extracted after treatment for further analyses. Treatment with inflammatory cytokines, IL-1β and TNF-α, but not IL-6, significantly increased GLT-1 steady-state levels ($p \leq 0.05$) without affecting mRNA levels, suggesting the cytokine-induced GLT-1 was regulated through post-transcriptional modifications. Among the candidate microRNAs predicted to modulate GLT-1, only microRNA-181a was significantly decreased following the IL-1β treatment ($p \leq 0.05$). Co-treatment of microRNA-181a mimic in IL-1β-treated primary astrocytes and neurons effectively blocked the IL-1β-induced upregulation of GLT-1. Lastly, we attempted to determine the link between GLT-1 and microRNA-181a in human AD brains. A significant reduction of GLT-1 was found in AD hippocampus tissues, and the ratio of mature microRNA-181a over primary microRNA-181a had an increasing tendency in AD. MicroRNA-181a controls rapid modifications of GLT-1 levels in astrocytes. Cytokine-induced inhibition of microRNA-181a and subsequent upregulation of GLT-1 may have physiological implications in synaptic plasticity while aberrant maturation of microRNA-181a may be involved in pathological consequences in AD.

Keywords: Alzheimer's disease, cytokines, GLT-1, glutamate excitotoxicity, microRNA

INTRODUCTION

Glutamate is a major excitatory neurotransmitter in the central nervous system and plays an important role in modulating synaptic plasticity, long-term potentiation (LTP), and learning and memory functions in the hippocampus. Glutamate release, reuptake, and recycling are tightly executed by neurons and astrocytes at tripartite synapses to evoke proper post-synaptic signal transduction and to prevent extensive leakage of glutamate beyond the synaptic cleft. In Alzheimer's disease (AD), glutamate dyshomeostasis is presented by the functional loss of excitatory amino acid transporter 2 (EAAT2) and elevation of glutamate in the cerebrospinal fluid [1–4]. Extensive alterations of glutamatergic pre-synaptic boutons is also observed in a pathology-dependent manner and correlates well with the

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severity of cognitive decline [5, 6] Specifically, impairment of glutamate reuptake results in elevated levels of synaptic or extrasynaptic glutamate and aberrant activation of synaptic and extrasynaptic N-methyl-D-aspartate receptors (NMDARs), leading to increased amyloid-β (Aβ) production [7, 8], which further promotes the release of glutamate and triggers synaptic damage [9]. In addition, mislocalization of tau in glutamategic synapses is shown to potentiate NMDAR-dependent Aβ neurotoxicity and synaptic loss [10]. These findings strongly implicate that the loss of glutamatergic synapses is a convergence point that links neuropathological signatures and clinical manifestations, further supporting the causal role of glutamate dyshomeostasis in AD.

Experimental models specifically highlight EAAT2 dysfunction as a critical contributor to AD. Genetic ablation of the glutamate transporter 1 (GLT-1), a mouse homologue of EAAT2, in a mouse model of AD exacerbates cognitive decline, in part recapitulating the above-mentioned observations in AD [11]. We and others have recently shown that Aβ species significantly reduces GLT-1 expression in the plasma membrane of astrocytes [9, 12–14], and pharmacological or genetic restoration of GLT-1 ameliorates Aβ or tau neuropathology and rescues cognition in AD mouse models [13, 14]. Together, these studies demonstrate that functional GLT-1 in mouse or EAAT2 in human (herein collectively referred to as GLT-1) is a critical factor for the survival of glutamatergic synapses. Thorough understanding of the regulatory mechanisms of GLT-1 expression will help elucidate the role of glutamate transporter in physiological and pathological conditions.

Recent studies identified several cytokines as major mediators of GLT-1 expression. This regulation is particularly important during physiological conditions and normal brain functions as spatially-limited transient elevation of pro-inflammatory cytokines, such as IL-1β, TNF-α, and IL-6, has been shown to modulate LTP in spatiotemporal manner [15]. While these cytokines are chronically elevated and dysregulated in AD [16] contribution of acute or chronic input of cytokines to astrocytes and underlying mechanisms to control GLT-1 expression remain largely unknown. Our previous findings of an age-dependent decrease of GLT-1 [14] in parallel with an increase of microRNA-181a (miR-181a) that significantly down-regulates several synaptic proteins involved in plasticity in 3xTg-AD mouse model [17] led us to hypothesize that miR-181a is a key mediator in plasticity of glutamatergic synapses by controlling the expression of synaptic proteins including GLT-1 in astrocytes. MicroRNAs are approximately 22 nucleotides noncoding RNAs that bind to the 3'-UTR or coding regions of their respective mRNA target to control gene expression at the post-transcriptional level by modulating mRNA translation and its stability [18]. Alterations of multiple microRNAs have been implicated in AD patients and may contribute to perturbing synaptic function and plasticity [19]. Thus, this study investigates the role of inflammation and microRNAs in controlling GLT-1 expression.

The aim of this study was to determine the mechanism that controls GLT-1 levels in astrocytes. When murine primary astrocytes and neurons were treated with IL-1β for 48 hours, the steady-state levels of GLT-1 were significantly upregulated while GLT-1 mRNA was relatively unaffected. Among multiple predicted microRNAs targeting GLT-1 mRNA, we determined that miR-181a was significantly and concomitantly decreased in cytokine-treated co-culture. Co-treatment of miR-181a mimics with IL-1β effectively blocked IL-1β-induced increase in GLT-1. Application of miR-181a mimics alone sufficiently suppressed GLT-1 in a concentration dependent manner, confirming the role of miR-181a in silencing GLT-1 expression. Lastly, we examined the potential involvement of miR-181a in AD by analyzing GLT-1 and miR-181a in postmortem hippocampal tissues from AD patients. We found that GLT-1 was significantly decreased in AD brains, and the ratio of mature miR-181a over primary miR-181a was elevated, though not significant from controls. Our results reveal that the expression of miR-181a regulates GLT-1 in astrocytes, and increased mature miR-181a may contribute to the loss of GLT-1 in AD.

MATERIALS AND METHODS

Primary astrocyte and neuron co-culture

As described previously [14], primary astrocytes were extracted from the cortex and hippocampus of postnatal day 2-3 (P2-P3) mice from wildtype (WT) mice. Primary cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 50 units/mL penicillin and 50 μg/mL streptomycin (P/S). Primary neurons were extracted from embryonic day 14–16 (E14-16) of WT mice. Primary neurons were added to confluent primary astrocytes in Neurobasal media with 2% B27 supplement, glutamine, and 5% FBS (plating

media) for the first 24 h then the medium was changed to media of similar contents but with only 2.5% FBS (growth media). Primary cells were treated after day 7 (of neuron addition). The purity of primary astrocytes and the presence of neurons were consistently monitored by IF staining with GFAP and Tau5 respectively.

Cell cultures

Naturally secreted Aβ monomers and oligomers were obtained from the conditioned medium (CM) of 7PA2 CHO cells that express the V717F AD mutation in APP_{751} (an APP isoform that is 751 amino acids in length, a kind gift from Dr. Edward Koo, UCSD) [20]. Both control CHO cells and 7PA2 cells were grown in the DMEM containing 10% FBS and P/S until ∼90% confluency. Cells were washed and medium replaced with neuronal growth media (described above) for ∼18 h. CM was collected and centrifuged at 1,000 × g for 10 min at 4°C to remove cell debris then used for treatment of astrocyte and neuron primary cell co-culture. CM from CHO cells and fresh growth media were used as controls. Recombinant Human Interleukin 1, beta (IL1β; PHC0815 ThermoScientific, CA, USA).

Quantitative measurement of secreted cytokines via cytokine multiplex assay

Cytokines from CHO-CM, 7PA2-CM or growth media (NB) were quantified before and after 48 h incubation with primary neuron and astrocyte co-cultures. The V-PLEX Proinflammatory Panel 1 (mouse) kits from Meso Scale Diagnostics (MSD, Gaithersburg, MD, USA) was used. The assay was performed according to the manufacturer's instructions and plates were analyzed on the MESO Quickplex SQ 120 (MSD). All standards and samples were measured in duplicate.

Western blot

Protein was extracted from primary murine astrocyte and neuron co-cultures using MPER while TPER was used to extract protein from human hippocampal tissue (Thermo Scientific, CA, USA). Bradford protein assay determined protein concentrations of MPER- or TPER- soluble fractions. Protein extracts were subsequently immunoblotted with the following antibodies: GLT-1 (a kind gift from Dr. Jeffrey David Rothstein, Johns Hopkins University), IL1-β (Biovision, CA, USA), tubulin and GAPDH (Abcam, MA, USA) were used to control for protein loading or to confirm no cross-contamination of each fraction. Band intensity was measured using the Odyssey Image station and Image Studio (version 2.1, Li-Cor Biosciences, NE, USA) and normalized by corresponding loading control protein.

RNA isolation, reverse transcription, and real-time polymerase chain reaction (RT-PCR)

Total RNA was isolated from primary cells or human hippocampal tissue using Direct-zol RNA MicroPrep and Direct-zol RNA Miniprep respectively according to manufacturer's protocol (Zymo Research Corp, CA, USA). Total RNA concentrations were determined using a spectrophotometer (NanoDrop Lite, ThermoScientific, CA, USA). Purity of samples was assessed with 2100 bioanalyzer (Agilent, CA, USA). cDNA was produced from 1000 ng RNA using NCODE Vilo cDNA synthesis kit (microRNAs) or SuperScript III First-Strand Synthesis kit (mRNAs) following manufacturer's protocol (Life Technologies, CA, USA). SYBR green detection for RT-PCR detailed protocol described previously [14, 17]. The TargetScan prediction software was utilized to identify microRNAs that had conserved 8mer, 7mer, or 6mer target sites on the 3' UTR of SLCI1A2.

TaqMan detection

cDNA was produced from 10 ng RNA using TaqMan MicroRNA Reverse Transcription Kit (mature miRNA; Catalog# 4366596, ThermoFisher, CA, USA) or High-Capacity cDNA Reverse Transcription Kit (primary miRNA; Catalog# 4368814, ThermoFisher, CA, USA). Quantitative RT-PCR was performed using CFX Connect Real-Time System (Bio-Rad, CA, USA) with the following TaqMan® miRNA assays (ThermoScientific, CA, USA): hsa-miR-181a (assay ID: 000480), has-miR-181b (assay ID: 001098), U6 snRNA (control; assay ID: 001973), hsa-mir-181a-1 (assay ID: Hs03302966_pri), hsa-mir-181a-2 (assay ID: Hs03302899_pri), human 18S (assay ID: Hs99999901_s1), mmu-mir-181b-1 (assay ID: Mm03307120_pri), mmu-mir-181a-2 (assay ID: Mm03306417_pri), and mouse Gapdh (assay ID: Mm99999915_g1). Cycling for PCR amplification was as follows: enzyme activation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and at 60°C for 60 s.

MiR-181a mimic transfection

Lipofectamine® RNAiMAX (Life Technologies, CA, USA) was utilized to transfect primary neuron and astrocyte co-culture with 15 nM, 30 nM, or 70 nM mirVana hsa-miR-181a miRNA mimic or mirVana Negative Control #1 miRNA mimic (Ambion, TX, USA) per manufacturer's protocol. Protein or RNA was extracted 48 h after transfection. To show that miR-181a mimics were upregulated in our cell culture after transfection, we extracted miRNAs after exposure and found higher levels of miR-181a in miR-181a (mimic) treated cells than the controls. Different concentrations of miR-181a were tested based on previous transfection reports [17, 21].

Statistics

All quantitative data are expressed as mean ± SEM. Data analyses were obtained using unpaired, two-tailed *t* test or One-way ANOVA followed by *post hoc* tests (Holm-Sidak or Dunn's multiple comparison test). The data were analyzed using Prism (GraphPad Prism Software) and values $p \leq 0.05$ were considered significant.

RESULTS

Inflammatory cytokines, IL-1β or TNFα, upregulate GLT-1 in astrocytes

We previously reported that conditioned media containing naturally secreted $A\beta_{40}$ and $A\beta_{42}$ species from 7PA2 cells (7PA2-CM) significantly downregulated the steady-state levels of GLT-1 compared to those from CHO cells lacking Aβ species (CHO-CM) in primary astrocytes and neuronal co-culture [14]. While investigating a potential molecular mechanism, we found that GLT-1 was significantly elevated in primary astrocyte and neuronal co-cultures treated with CHO-CM by 47% while 7PA2-CM still significantly suppressed GLT-1 steady state levels by 36% compared to those in neurobasal media (control) (Fig. 1). To examine the rise in GLT-1 levels from CHO-CM compared to control, we hypothesized that cytokines may be involved in GLT-1 regulation since they have previously been shown to modulate GLT-1 expression [22, 23]. We tested a panel of cytokines before and after the 48-h treatment on our primary astrocytes and neuronal co-culture through MSD. While no significant differences were detected in selected cytokine levels among control, CHO-CM, and 7PA2-CM prior to the incubation (data not shown), significantly elevated levels of pro-inflammatory cytokines, IL-1β, TNF-α, IL-6, IL-5, and IL-10 were found in CHO-CM, but not in control or 7PA2-CM, after the 48-h treatment with the co-culture (Fig. 2A-E). No changes were observed in IL-12p70, IFN-γ, IL-2, and IL-4 (data not shown).

We then examined whether major pro-inflammatory cytokine, IL-1β, TNFα, or IL-6, alone could modulate the levels of GLT-1. Previous reports have shown independent cytokines differentially regulate GLT-1 in a dose dependent manner [22, 24] thus based on these reports we chose 20 ng/mL as the concentration in our treatment paradigm. Treatment with recombinant IL-1β or TNF-α (20 ng/ml) significantly increased GLT-1 levels by 35% and 69%, respectively while recombinant IL-6 did not (Fig. 3). Cytokine treatment did not alter GLT-1 mRNA measured by qRT-PCR (data not shown). These results show that, in our treatment paradigm on primary neuron and astrocyte co-cultures, specific cytokines upregulate GLT-1 steady state levels while having no significant effect on GLT-1 mRNA.

MiR-181a mediates IL-1β-induced GLT-1 upregulation in astrocytes

We continued to investigate the underlying molecular mechanism by which IL-1β upregulated GLT-1 in the primary co-culture system. MicroRNAs regulate gene expression post-transcriptionally, and several microRNAs have been reported to modulate GLT-1 expression [21, 25, 26]. Based on these reports and results from Targetscan prediction search, we selected 5 candidate microRNAs (20a, 29a, 107, 124a, and 181a) to screen for IL-1β-induced GLT-1 upregulation. Among these candidate miRNAs, we only found that miR-181a was significantly reduced by 34% after 48 h of 20 ng/mL IL-1β treatment in primary astrocyte and neuron co-culture compared to the untreated control (Fig. 4A). In addition, we quantify primary miR-181a in these samples. In the mouse, the miR-181 family consists of four mature microRNAs (miR-181a, miR-181b, miR-181c, and miR-181d) with miR-181a being transcribed from two genes *pri-miR-181ab1* and *pri-miR-181ab2* [27, 28]. No significant differences were detected in the expression of pri-miR-181a-1 and pri-miR-181a-2 between the treatments (Fig. 4B, C) indicating that IL-1β directly regulates the levels of mature miR-181a.

We then determined whether miR-181a directly suppressed GLT-1 steady state levels *in vitro*. We

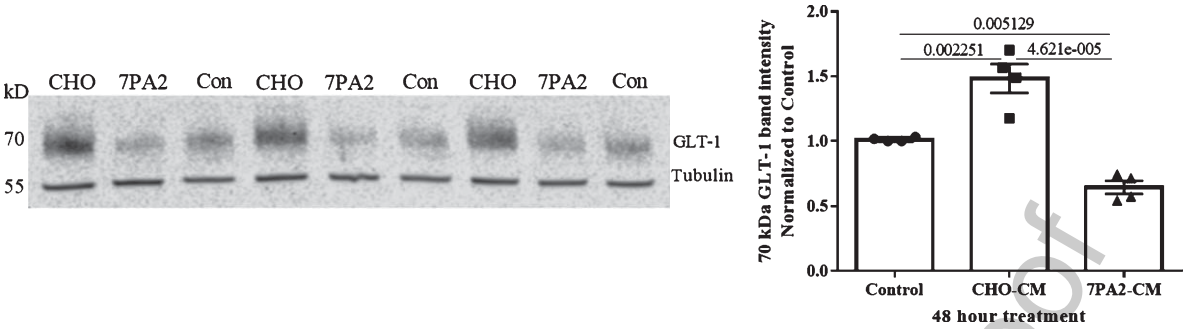

Fig. 1. GLT-1 steady state levels altered after 48 h exposure to CHO-CM or 7PA2-CM compared to control. Primary astrocyte and neuron co-culture treated with CHO-CM elevated GLT-1 steady state levels while 7PA2-CM decreased GLT-1 steady state levels relative to regular neurobasal growth media (control) after 48 h treatment. GLT-1 mean ± std. error: Control = 1.01 ± 0.00816, CHO-CM = 1.48 ± 0.111, 7PA2-CM = 0.645 ± 0.0506, Control versus CHO $p = 0.0023$, Control versus 7PA2-CM $p = 0.0051$ and CHO-CM versus 7PA2-CM $p < 0.0001$, $n = 4$ independent experiments in triplicates, and each dot represents mean of independent experiment. $p \leq 0.05$ considered significant by one-way ANOVA followed by Holm-Sidak's *post hoc* multiple comparisons test. Abbreviations: Con = Control; neurobasal growth media; CHO = Chinese hamster ovary; 7PA2 = CHO cells that express the V717F AD mutation in APP_{751} (an APP isoform that is 751 amino acids in length); CM = conditioned media; GLT-1 = glutamate transporter 1.

transfected miR-181a mimics to primary astrocyte and neuron co-culture for 48 h and found miR-181a mimics decreased the steady-state levels of GLT-1 by 32% in a concentration-dependent manner compared to the vehicle (Fig. 4D). We confirmed that cells transfected with 70 nM miR-181a contained higher levels of miR-181a specifically than the control via RT-PCR (44 fold higher than control). Accordingly, we co-treated the co-culture with 20 ng/mL IL-1β and 70 nM miR-181a mimic for 48 h, and found miR-181a mimics counteracted the effect of IL-1β reducing GLT-1 steady-state levels by 48% compared to 20 ng/mL IL-1β treatment alone (Fig. 4E). These results show IL-1β as an external source to downregulate miR-181a in cells to increase GLT-1 expression. To further explore whether the observed upregulation of GLT-1 by CHO-CM (Fig. 1) was primarily mediated by the downregulation of miR-181a, we quantified both primary and mature miR-181a following CHO-CM or 7PA2-CM treatment in primary co-culture preparations. We did not find any significant differences in the levels of mature miR-181a, pri-miR181a-1, pri-miR-181a-2 or the ratios of mature/pri-miR-181a-1 and mature/pri-miR-181a-2 in CHO- and 7PA2-CM compared to the control (data not showed), suggesting that other factors (besides IL1-β) may modulate miR-181a expression in co-culture samples treated with CHO-CM.

Loss of GLT-1 in AD brain and a possible contribution of mature miR-181a

The loss of GLT-1 has been observed in post-mortem AD brains [2, 4]. To examine whether the loss of GLT-1 in AD was mediated in part by miR-181a, we assessed primary and mature miR-181a expression and GLT-1 steady-state levels in hippocampal tissues from AD patients and age-matched cognitively normal individuals (Table 1 for patient information). We show that GLT-1 steady-state levels were significantly reduced in AD brains by 72% (Fig. 5A), consistent with previous reports [2, 4]. In addition, we observed statistical significant higher levels of pro-IL-1β in AD samples, consistent with the well-known elevation in inflammatory responses in AD brains (Fig. 5B). To correlate the loss of GLT-1 with increased mature miR-181a, we quantitatively measured both mature and primary miR-181a in these tissues. Like in the mouse, the human miR-181 family consists of four mature microRNAs (miR-181a, miR-181b, miR-181c, and miR-181d) with miR-181a being transcribed from *pri-miR-181ab1* gene in chromosome 1 and *pri-miR-181ab2* gene in chromosome 2 [27, 28]. While the mean of mature miR-181a between non-demented individuals and AD was not different (Fig. 5C) we found lower levels of pri-miR-181a-1 in AD brains compared to age matched controls by 46% (Fig. 5D) but not pri-miR-181a-2 (Fig. 5F) suggesting that, overall, less miR-181a transcript is being produced in AD patients. Consequently, the ratio of mature miR-181a over immature form had an increasing trend in AD patients compared to controls (Fig. 5E, G).

DISCUSSION

We report that miR-181a plays a pivotal role in post-transcriptional modulations of GLT-1 in astro-

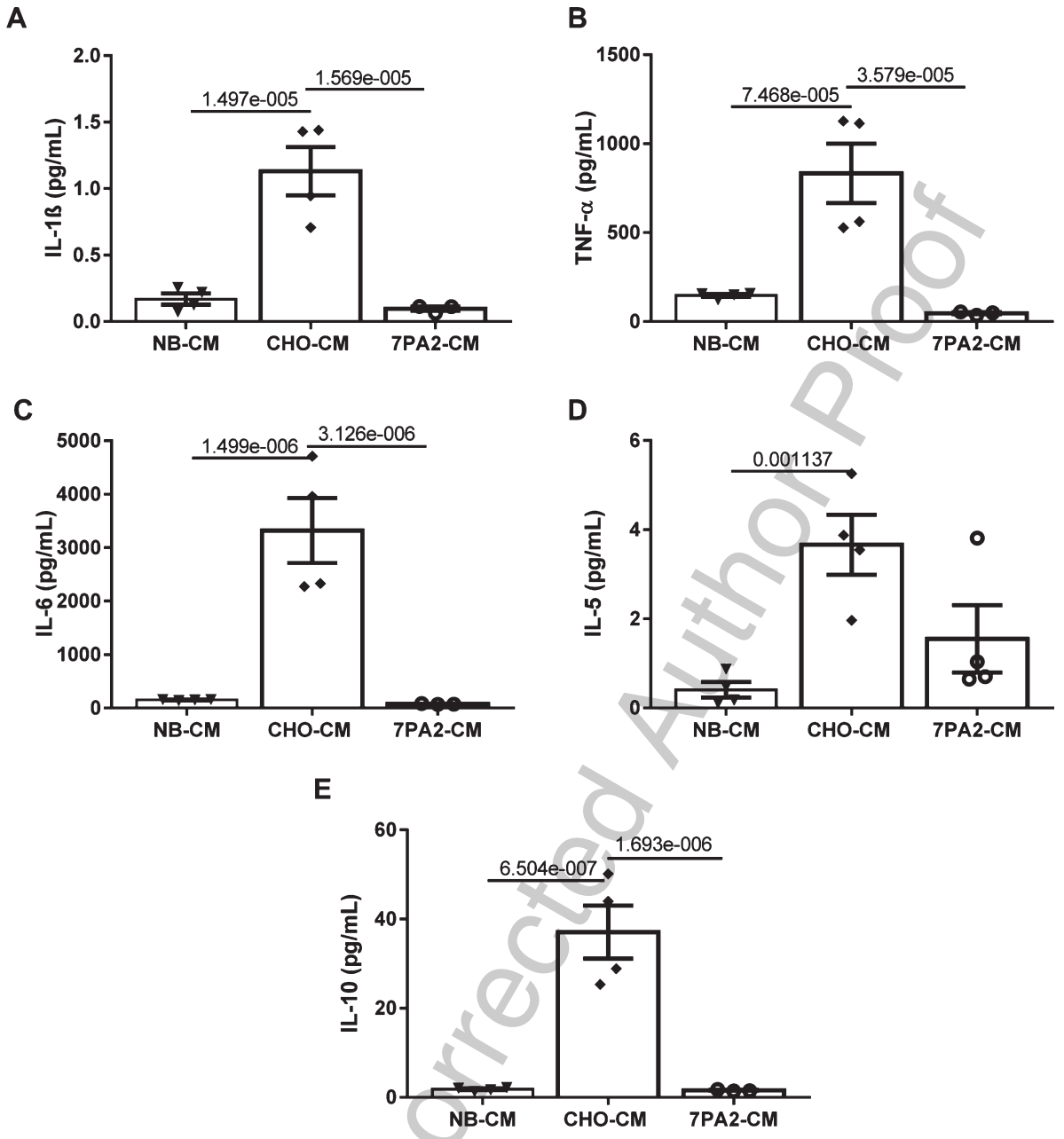

Fig. 2. Significant differences in cytokine profile between CHO- and 7PA2-CM after 48 h of exposure to co-culture. (A) IL-1β levels in pg/mL (mean ± std. error: Control = 0.170 ± 0.0422, CHO-CM = 1.13 ± 0.182, 7PA2-CM = 0.0969 ± 0.0165), (B) TNF-α (Control = 147 ± 8.17, CHO-CM = 833 ± 166, 7PA2 = 46.5 ± 5.92), (C) IL-6 (Control = 154 ± 4.79, CHO-CM = 3320 ± 607, 7PA2 = 75.2 ± 5.03), (D) IL-5 (Control = 0.407 ± 0.174, CHO-CM = 3.66 ± 0.675, 7PA2 = 1.55 ± 0.759), and (E) IL-10 (Control = 1.82 ± 0.199, CHO-CM = 37.1 ± 5.94, 7PA2 = 1.57 ± 0.108). n = 3-4 independent experiments in duplicates, and each dot represents mean of independent experiment. $p \leq 0.05$ considered significant by one-way ANOVA followed by Sidak's *post hoc* multiple comparison's test.

cytes. IL-1β and TNF-α, but not IL-6, significantly upregulated GLT-1 levels, and particularly IL-1β-induced GLT-1 was mediated by down-regulation of miR-181a in the primary neuron and astrocyte co-culture. Lastly, we examined whether miR-181a was involved in pathological loss of GLT-1 in AD. In agreement with previous studies [2, 4, 29], GLT-1 decreased in the hippocampus from advanced stages of AD patients compared to age-matched non-demented individuals. In these tissues, we found a

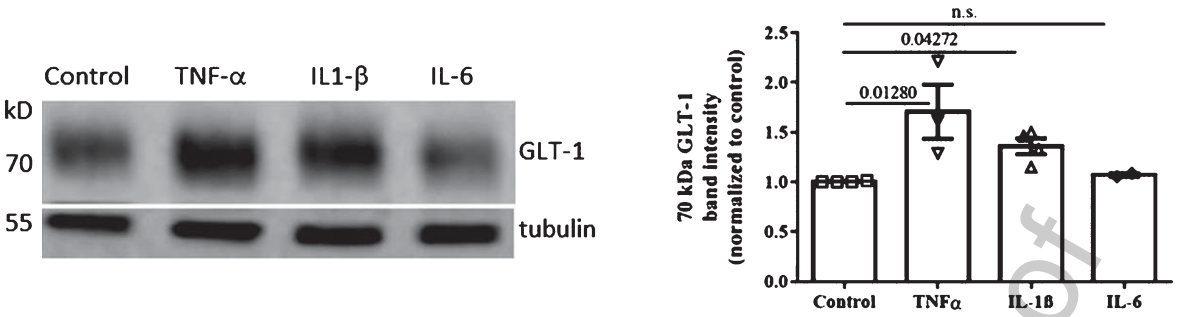

Fig. 3. Synthetic recombinant 20 ng/mL IL-1β or TNF-α increase GLT-1 but not IL-6 after 48 h exposure compared to control. GLT-1 mean ± std. error: Control = 1.01 ± 0.00332, TNF-α = 1.71 ± 0.271; $p = 0.0128$, IL-1β = 1.36 ± 0.0797; $p = 0.0427$, IL-6 = 1.07 ± 0.0172; $p > 0.9999$. $n = 2$–4 independent experiments in duplicates or triplicates, and each dot represents mean of independent experiment. $p \leq 0.05$ considered significant by one-way ANOVA followed by Dunn's *post hoc* multiple comparison's test.

trend toward increasing ratio of mature miR-181a over primary miR-181a in AD brains when compared to age-matched control brains, suggesting that aberrant maturation of miR-181a could be early pathological changes during the disease course.

IL-1β-mediated GLT-1 regulation may elicit a wide spectrum of effects because of the involvement of various pathways and duration of exposure. In comparison to our results, others have shown that IL-1β decreases GLT-1 [22], however this dichotomous effect on GLT-1 may be in part owing to the concentration of cytokines [15], diversity of models (single versus co-cultures) [30], exposure time [15, 30], serum use [31], and differential regulation between protein and mRNA. Specifically, the binding of IL-1β to the IL-1 receptor 1 (IL-1R1) followed by recruitment of accessory protein subunit expressed on astrocytes can ultimately activate transcription factors including NF-κB [32, 33]. NF-κB in turn can bind to multiple binding sites available on the GLT-1 promoter; however, distinct pathways can repress or activate GLT-1 expression depending on the co-factors present [23, 34]. Further complicating a succinct conclusion among reports but indicating a need to explore other regulatory pathways mediated by IL-1β. Thus, we explored and convincingly show that IL-1β regulates GLT-1 post-transcriptionally via microRNAs.

The duration of our experimental conditions performed in our *in vitro* studies is acute and not in the chronic state of IL-1β treatment that mimics the disease state commonly observed in AD. In this regard, our experimental condition may represent in part a physiological state with transient elevation of IL-1β and other local cytokines. Such acute treatment of cytokines, specifically IL-1β, may relay essential signals from local microenvironment to astrocytes to adapt their functions. In the presence of astrocytes, GLT-1 upregulation can be a compensatory mechanism to prevent sustained NMDAR activity indicating a neuron-glia crosstalk. As others have demonstrated that IL-1β increases activity of NMDAR in a manner sufficient to increase neuronal cell death in neuronal cultures [35]. The effect of IL-1β seen in our treatment paradigm on astrocytes and neurons may be to control glutamate levels by modulating astrocytic GLT-1 expression at the synapse.

While IL-1β has been shown to suppress LTP on primary neuronal cultures and hippocampal slices [15, 36–38], other studies show that IL-1β is important and essential for LTP. Specifically, IL-1R1 deficient mice develop memory impairment and/or LTP inhibition [39, 40]. This impairment was rescued by introducing wildtype astrocytes in the IL-1R1 deficient mice [41]. Moreover, IL-1β expression significantly increases following LTP and learning [42]. In addition, GLT-1 is responsible for glutamate uptake during LTP and pharmacological inhibition of astrocytic GLT-1 activity reduced LTP and prevented induction of additional LTP in hippocampal slices and cell culture experiments [43], suggesting that GLT-1 plays an important role in synaptic plasticity. MiR-181a is enriched in neurons and astrocytes particularly in the hippocampus and is critically involved in synaptic plasticity and memory processing [17, 27, 44]. Recent growing bodies of evidence strongly implicate spatiotemporal maturation of precursor microRNA to mature miR-181a at the synaptic compartment during low-frequency stimulation, which subsequently down-regulates CAMKII [44]. Other critical plasticity-related proteins regulated by miR-181a include cFos and SIRT1 [17, 44]. Thus, at the tripartite synapse, the activity-dependent maturation of miR-181a rapidly changes synaptic constituents

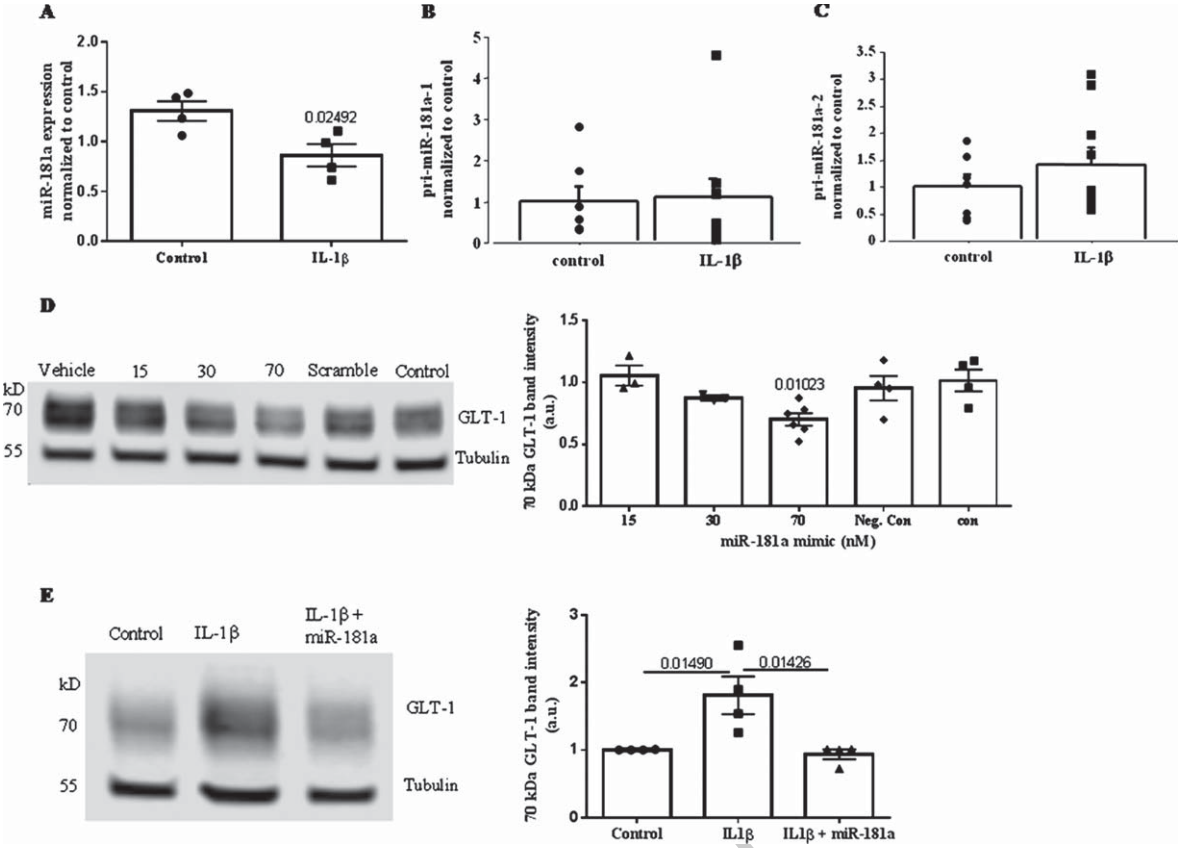

Fig. 4. IL-1β increases GLT-1 steady state levels via miR-181a. (A) TaqMan® Chemistry for RT-PCR using miR-181a primer shows 48 h exposure of 20 ng/mL IL-1β downregulates miR-181a; GLT-1 mean ± std. mean error: Control = 1.31 ± 0.0980 and IL-1β = 0.864 ± 0.113, $p = 0.0249$. $n = 4$ independent experiments in triplicates, and each dot represents mean of independent experiment. $p \leq 0.05$ considered significant by unpaired *t*-test. (B) No significant differences were observed in mmu-pri-miR-181a-1 (Control = 1 ± 0.35 and IL-1β = 1.08 ± 0.46, $p = 0.89$) or mmu-pri-miR-181a-2 (Control = 1 ± 0.21 and IL-1β = 1.41 ± 0.29, $p = 0.32$) (C). (D) miR-181a mimic decreases GLT-1 expression compared to the vehicle in a concentration-dependent manner after 48 h. GLT-1 mean ± std. error: Vehicle = 1.03 ± 0.0102, Control = 1.02 ± 0.0882, 15 nM = 1.056 ± 0.0813, 30 nM = 0.875 ± 0.0149, 70 nM = 0.701 ± 0.0509 ($p = 0.0102$), negative control = 0.953 ± .0983. $n = 5$ independent experiments with duplicates or triplicates, and each dot represents mean of independent experiment. $p \leq 0.05$ considered significant by one-way ANOVA followed by Dunn's *post hoc* multiple comparison's test. (E) Co-treatment of 70 nM miR-181a mimic with 20 ng/mL IL-1β after 48 h returned GLT-1 steady state levels relatively close to control levels. GLT-1 mean ± std. error: Control = 1.00 ± 0.00241, IL-1β = 1.81 ± 0.280, IL-1β and miR-181a mimic = 0.935 ± 0.0705; Control versus IL-1β ($p = 0.0149$), IL-1β versus IL-1β and 70 nM miR-181a mimic ($p = 0.0143$). $n = 4$ independent experiments in duplicates or triplicates, and each dot represents mean of independent experiment. $p \leq 0.05$ considered significant by one-way ANOVA followed by Holm-Sidak's *post hoc* multiple comparison's test. miR-181a mimic = mirVana hsa-miR-181a-5p and scramble = mirVana Negative Control #1 microRNA mimic.

Table 1
Human samples information

Neuropathology Dx	Mean age (y)	MMSE	Plaque Stage	Tangle Stage	*n**
Control	83 – 87	22 – 30	A-C	3–5	6 – 8
AD	80 – 90	10 – 17	A-C	4–6	6 – 8

*equal number of males and females.

and post-synaptic signaling cascades. This body of work shows that IL1-β, GLT-1 and miR-181a are all important regulators of synaptic plasticity. Together with our results presented herein the interesting possibility emerges that IL-1β-mediated upregulation of GLT-1 via downregulation of miR-181a may be part of the mechanisms involved in LTP and synaptic plasticity. Augmented levels of GLT-1 would be required to deal with the increased activity associated with strengthened synapses and to avoid neurotoxicity. However, this hypothesis remains to be explored and further studies are needed to validate our results under

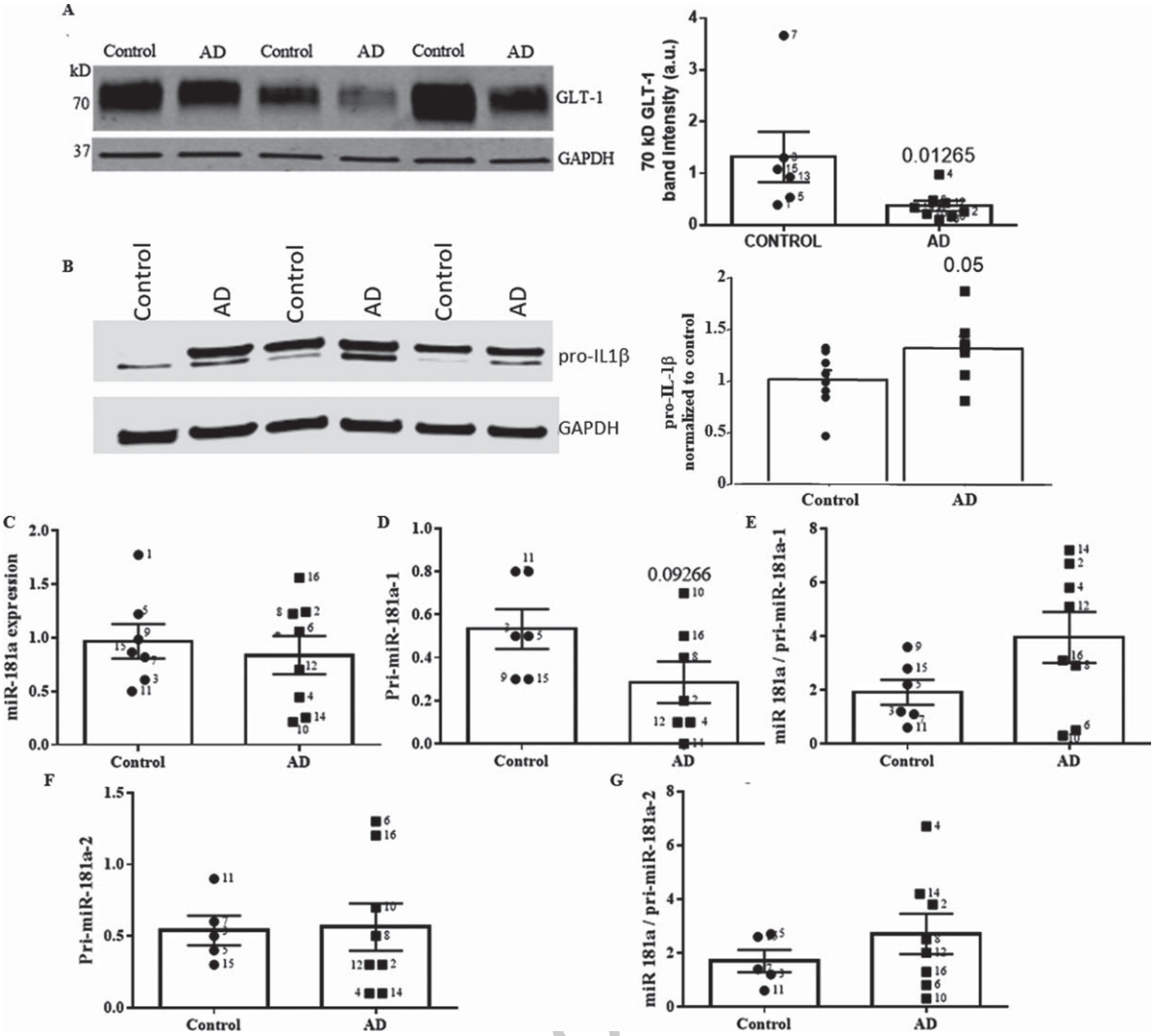

Fig. 5. GLT-1, pro-IL-1β, and miR-181a levels in hippocampal human samples. GLT-1 steady state levels and microRNA levels from hippocampal human AD patients compared to age-matched controls. A) GLT-1 steady state levels significantly decrease in AD human patients compared to age-matched human controls. GLT-1 mean ± std. mean error: Control = 1.32 ± 0.489 and AD = 0.370 ± 0.0975 ($p = 0.0127$). B) pro-IL-1β steady state levels were significantly increased in AD human patients compared to age-matched human controls. Pro-IL-1β control = 1 ± 0.09 and AD = 1.3 ± 0.10 ($p = 0.05$). C) No significant differences between mature miR-181a levels from AD patients compared to age-matched control. miR-181a mean ± std. mean error: Control = 0.967 ± 0.162 and AD = 0.837 ± 0.179 ($p = 0.7789$). D) Decrease of immature miR-181a levels in AD compared to age matched control. pri-miR-181a-1 mean ± std. mean error: Control = 0.533 ± 0.0919, AD = 0.2857 ± 0.0962 ($p = 0.0927$). E) Ratio of mature miR-181a and pri-miR181a-1 is higher in AD compared to age matched control groups. Control = 1.92 ± 0.469, AD = 3.95 ± 0.944 ($p = 0.228$). F) No differences in pri-miR-181a-2 levels between AD and age matched controls. pri-miR-181a-2 mean ± std. mean error: Control = 0.540 ± 0.103, AD = 0.563 ± 0.166 ($p = 0.758$) and (G) ratio of miR-181a and pri-miR-181-a-2 ± std. mean error: Control = 1.70 ± 0.410, AD = 2.70 ± 0.748 ($p = 0.622$). Each symbol represents 1 human sample with an ID corresponding to the number next to the symbol ($n_{total} = 5 - 8$ samples). $p \leq 0.05$ considered significant by unpaired *t*-test. Immature miR-181a corresponds to primary miR-181a.

relevant physiological conditions using appropriate *in vivo* models.

In addition to its role in dynamic physiological mechanisms, microRNAs are increasingly recognized as important biomarker for various diseases [45]. In AD, reduction of miR-181a was initially reported in the cerebrospinal fluid compared to the age-matched controls [46]. More recently, as a part of identification of plasma biomarkers for AD, miR-181a appeared as one of novel microRNAs that are significantly different when compared between control and MCI or early-stage AD, but not established

AD [47], suggesting that miR-181a may be involved in the prodromal or early stages of AD pathogenesis, possibly when synaptic abnormalities are being triggered. We attempted to determine the levels of mature miR-181a in post-mortem AD brains. Although we detected an increasing trend of mature miR-181a ratio in AD, it failed to show statistical significance, possibly because these brains were from relatively advanced stages of AD. Further studies will be needed to extensively analyze the microRNA profile in brain tissues from MCI and early stages of AD patients.

Conclusion

Insurmountable evidence of GLT-1's importance in maintaining a healthy microenvironment in normal aging brains and the consequences that may result from its dysfunction has led to GLT-1 being a potential target for therapeutic interventions for AD. Our approach simplifies a very complex system but has allowed us to focus on important components that unveil a potential molecular mechanism that affect GLT-1 steady state level expression. Our *in vitro* study is a primary step to unveiling a potential and important molecular mechanism that regulates GLT-1 steady state levels. Further studies are needed to extrapolate our results and apply them in a more complex model. In conclusion, we provide a beneficial role of inflammatory cytokines and suggest that this may be a defense mechanism against a possible neurotoxic environment.

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