



Differential regulation of C-terminal splice variants of the glutamate transporter GLT-1 by tumor necrosis factor- α in primary cultures of astrocytes

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ABSTRACT

The high-affinity glutamate transporter GLT-1 plays a key role in the control of the glutamate homeostasis in the central nervous system and protects neurons against excitotoxicity. Splice variants of the original transcript have been identified and their involvement in neurodegenerative disorders has been proposed. However, the functions and the regulations of these isoforms remain unclear. In this study, we focused our interest on the expression of two C-terminal splice variants of GLT-1 (GLT-1a and b) in primary astrocyte cultures exposed to distinct chemical environments. While GLT-1a and GLT-1b mRNAs were both increased in response to treatment with N⁶,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate (dBcAMP), the culture supplement G5 or tumor necrosis factor- α (TNF- α), the regulation of GLT-1b appeared quicker and was more pronounced. Besides, using validated antibodies, we evidenced a differential regulation of the two proteins in cells exposed to TNF- α . Thus, while dBcAMP and the G5 supplement stimulated the expression of both isoforms at 3 and 7 days, a transient upregulation of GLT-1a was induced by TNF- α , which contrasts with the sustained induction of the GLT-1b isoform. These results shed light on the complex influence of the pro-inflammatory cytokine TNF- α on GLT-1a mRNA and protein expression and on the necessity to distinctly consider the GLT-1 isoforms with appropriate tools in studies addressing the regulation of glutamate transporters.

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1. Introduction

The excitatory amino acid transporter 2 (EAAT2 in human and designated as GLT-1 in rodents) is the major glutamate transporter in the central nervous system (CNS) where it is predominantly expressed by astrocytes (Danbolt, 2001). Together with other glutamate transporters, EAAT2/GLT-1 contributes to the active clearance of glutamate from the synapses, ensuring controlled excitatory transmission and efficient neuroprotection against excitotoxicity. Late in the 90s, alternative splicing of the EAAT2/GLT-1 transcripts has been evidenced and a link between altered expression of the related variants and neurodegenerative diseases was proposed. Thus, elevated levels of exon7 and exon9-skipping isoforms of EAAT2 have been documented in the spinal cord of ALS patients (Lin et al., 1998; Meyer et al., 1999) and impaired glutamate uptake in Alzheimer disease has been associated with increased expression of the same transporter isoforms (Scott et al., 2010). Accordingly, combining the expression of EAAT2 lacking exon7 and exon9 with the native full length transporter has been recently shown to inhibit glutamate transport in *Xenopus* oocytes

(Scott et al., 2010). However, absolute measures of mRNA levels of these isoforms revealed their modest expression as compared to full length EAAT2 transcripts (Lauriat et al., 2007; Walton et al., 2007). In 2002, a C-terminally modified GLT-1 protein, designated as GLT-1b was identified (Chen et al., 2002). At variance with the original transporter (designated as GLT-1a), GLT-1b is detected in both neurons and astrocytes, elucidating contradicting data that evidenced dihydrokainate sensitive glutamate uptake activity in neurons (Berger et al., 2005; Chen et al., 2002). The distinct C-terminus of GLT-1b harbours a PDZ motive enabling the interaction with scaffolding proteins such as PSD-95 and PICK1, both involved in protein trafficking in the brain (Bassan et al., 2008; Gonzalez-Gonzalez et al., 2008, 2009). Indeed, GLT-1a and GLT-1b appears differentially addressed at the cell surface of cerebellar granule cells (Karatas-Wulf et al., 2009). Noteworthy however, co-immunoprecipitation assays performed on brain tissue samples or immunostaining studies on transfected cells have suggested that GLT-1a and GLT-1b associate as heteromers (Bassan et al., 2008; Gonzalez-Gonzalez et al., 2009; Peacey et al., 2009). Similarly, the other C-terminally distinct isoform GLT-1c, detected in the retina (Rauen et al., 2004), also contains a PDZ motive, but interacting partners have not been reported. Splicing has also been evidenced within the 5' sequence of GLT-1, giving rise to 4 N-terminally distinct GLT-1 isoforms (Utsunomiya-Tate et al., 1997; Rozyczka

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and Engele, 2005) which are all expressed at the cell surface and assemble into heteromers. Finally, alternative splicing occurs within untranslated sequences, likely influencing the stability of mRNA or the translation efficiency (for review, see Lauriat and McInnes, 2007).

Altered glutamate uptake is frequently observed in neurological disorders and the mechanisms controlling glutamate transporter expression have received considerable attention during the last decades. Best documented is the influence of growth factors and inflammatory mediators released by neighbour cells during CNS development or during nervous lesions, respectively (Danbolt, 2001). More specifically, the regulation of glutamate transporters by soluble factors has been also examined in models of astrocyte cultures. In this respect, it is noteworthy that even though alterations in the expression of selected GLT-1 isoforms have been documented in neurological disorders (Lauriat and McInnes, 2007), little is known regarding the regulation of individual splice variants in response to changes in the cell environment. Indeed, the commonly used experimental approaches fail to distinguish or to take into account all GLT-1 isoforms. Therefore, the aim of the present study was to characterize the expression of the two major C-terminal splice variants of GLT-1 in primary cultured astrocytes exposed to known modulators of the expression or activity of GLT-1. Thus, absolute quantifications of mRNA expression were combined with immunodetection of encoded proteins by validated antibodies specifically recognizing GLT-1a and GLT-1b.

2. Experimental procedures

2.1. Cell culture

Primary cultures of astrocytes from 2 to 3 days old Wistar rats were prepared as previously described (Vermeiren et al., 2005). Briefly, cortices were collected and meninges carefully removed. After mechanical dissociation, isolated cells were seeded and left to proliferate for 10 days in Dulbecco's Modified Eagle Medium (GlutaMAX, Invitrogen, Merelbeke, Belgium) supplemented with 10% foetal bovine serum (Thermoscientific, Tournai, Belgium), 50 µg/mL penicillin-streptomycin (Invitrogen), 2.5 µg/mL Fungizone® (Invitrogen) and L-proline (50 µg/mL) (Sigma-Aldrich). Non astroglial cells were removed by 24 h shaking in an orbital shaker at 200 rpm. At confluence, cells were trypsinized (trypsin 0.05%, Invitrogen) and plated at a density of 1.5×10^4 cells/cm². For immunocytochemistry, cells were seeded at a density of 5×10^3 cells/cm² on poly-L-lysine (Sigma-Aldrich) coated glass coverslips. Treatments of astrocytes were initiated 48 h after plating, with either tumor necrosis factor alpha (TNF-α), N⁶,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate sodium salt (dBcAMP) or the G5 supplement (100 mg/L biotin, 0.5 mg/L basic FGF, 1 mg/L EGF, 5000 mg/L human transferrin, 500 mg/L insulin, 0.36 mg/L hydrocortisone, 0.52 mg/L selenite), respectively purchased from AbD Serotec (Morphosys AbD GmbH, Düsseldorf, Germany), Sigma-Aldrich and Invitrogen. During treatment, medium was renewed every 3–4 days. Cells were daily observed using an Olympus CKX41 inverted microscope coupled to a PixelINK camera (Iris Vision B. V., Etten-Leur, The Netherlands).

COS-7 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% foetal bovine serum and 50 µg/mL penicillin-streptomycin and seeded at a density of 1.5×10^4 cells/cm² for transfection. Transfection was performed with Lipofectamine™ (Invitrogen), according to manufacturer's instructions.

2.2. RNA extraction and quantitative PCR (qPCR)

Total RNA was extracted from cell cultures using TriPure Isolation Reagent (Roche Applied Science, Vilvoorde, Belgium). After treatment with RNase-free DNase (Promega, AJ Leiden, The Netherlands) to avoid DNA contamination, RNA

was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Nazareth Eke, Belgium). qPCR was performed with the iQ5 Monocolor Real Time PCR detection system (Bio-Rad Laboratories), in conditions suggested by the manufacturer and previously validated (Tilleux et al., 2007). Briefly, each assay included 2 ng of cDNA from each sample, 0.375 µM of specific primers (from Invitrogen, listed in Table 1) and iQ SYBR Green Supermix (Bio-Rad Laboratories) in a total volume of 25 µL. GLT-1a and GLT-1b specific primers were designed using Primer3 (<http://frodo.wi.mit.edu/primer3/>) while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glial fibrillary acidic protein (GFAP) primers were previously validated.

The amplification protocol was conducted as follows: 30 s denaturation at 95 °C, 45 s annealing at 60 °C, and 15 s elongation at 79 °C for 40 cycles. The fluorescence signal was monitored at the end of each elongation step. For quantitative analyses, amplification was performed in the same conditions for each target gene with serial dilutions of a mix of the cDNA templates. For GFAP expression, each sample was normalized to the relative amplification of GAPDH. Absolute quantification of GLT-1a and GLT-1b expression, herein express as mRNA copies per µg of total RNA, was performed using dilutions of cloned cDNA sequence of these transcripts. Calculation of cycle threshold values, standard curves preparation, and relative quantifications of mRNA in the samples were performed using the post run data analysis software provided with the iCycler system (Bio-Rad Laboratories).

2.3. Western-blotting

Cells were collected in phosphate-buffered saline (PBS, Invitrogen) and centrifuged for 10 min at 8000 × g. Total protein extraction from cell pellets was performed in the solubilisation buffer [3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) 20 mM, Tris-HCl 10 mM pH 7.4, ethylenediaminetetraacetic acid (EDTA) 0.5 mM, DL-dithiothreitol (DTT) 1 mM, phenylmethanesulfonyl fluoride (PMSF) 0.5 mM, protease inhibitor cocktail (PIC) 1%]. For brain protein extraction, adult Wistar rats were killed by CO₂ asphyxia and brain tissues were homogenized in lysis buffer (Tris-HCl 20 mM pH 7.4, NaCl 100 mM, Triton X-100 1% and PIC 1%) using a Teflon potter. Cell debris were eliminated by centrifugation at 8000 × g during 10 min. Protein concentration in each sample was determined by Bradford method using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories). Each sample was further diluted in loading buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate (SDS) and 0.01% bromophenol Blue) and boiled for 10 min. Aliquots of 30–40 µg of proteins were separated through 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes. To reduce non-specific labelling, membranes were firstly incubated for 30 min at 22 °C in Tris-buffered saline (TBS) containing 5% skimmed milk and 0.05% Tween-20. For immunoblotting, membranes were incubated with the primary antibodies as followed: guinea pig polyclonal anti-GLT-1 (1:2500, Chemicon, Millipore, Brussels, Belgium), rat monoclonal anti-GLT-1b (1:1500) and rabbit polyclonal anti-actin (1:1000, Sigma-Aldrich). This specific anti-GLT1b monoclonal antibody directed against the C-terminus of the rat GLT-1b was generated in LOU/C rats which were immunized with the synthetic peptide H₂N-GPFPFLDIETCI-COOH corresponding to the C-terminal sequence of GLT-1b. Hybridomas were produced by fusing the non-secreting rat cell line IR983F (Bazin, 1982) with splenocytes from one of the immunized rat of which serum was specifically recognizing the peptide. Limiting dilutions of hybridoma cells permitted the isolation of clones and the specificity of the secreted antibody from individual clones was confirmed by ELISA against the synthetic peptide and excluded against a non-relevant peptide. Secondary antibodies were HRP conjugated, respectively: goat anti-guinea pig IgG (1:10,000, Chemicon, Millipore), mouse monoclonal anti-rat IgG (1:4000, Immunologie/Chirurgie Expérimentale, UCL, Belgium) and goat anti-rabbit IgG (1:3000, Sigma-Aldrich) and incubated 1 h at 22 °C. Immunoreactive proteins were visualized using chemiluminescence reaction on Amersham Hyperfilm™ ECL (GE Healthcare Europe GmbH, Diegem, Belgium). Densitometric analyses of the signals detected by autoradiography were performed using an MCID-M4 imaging system (Imaging Research, Ontario, Canada).

2.4. Immunocytochemistry

After washing with PBS, cells grown on coverslips were fixed for 15 min at 22 °C in ethanol 95% and then permeabilized in Triton X-100 1% for 15 min. To avoid non-

Table 1
PCR primers and amplification products (F, forward primer; R, reverse primer).

Target	Accession number	Primers	Primer position	Product size
GFAP	NM_017009	F: GGTAAGACGGTGGAGATGC R: GCACACCTCACATCATCC	1221–1241 1281–1301	80 bp
GAPDH	NM_017008	F: GTCTCTGTGACTTCAACAG R: AGTTGTCATTGAGAGCAATGC	913–933 968–989	76 bp
GLT-1a	NM_017215	F: TGTCTATGCCGCACACAAC R: TCCTCAACATCGAGTCAGC	1718–1738 1788–1808	90 bp
GLT-1b	NM_001035233	F: AATGTGTCTATGCCGCACAC R: GCAGGGGATGGTGCTTTT	1714–1734 1824–1842	128 bp

specific labelling, cells were firstly incubated for 30 min at 37 °C in blocking solution (PBS containing 3% skimmed milk) and then incubated for 1 h 30 min at 37 °C with the rabbit anti-GFAP (1:1000, Dako Belgium nv/sa, Heverlee, Belgium). Immunolabelling was revealed using the FITC-coupled anti-rabbit IgG (1:500, Jackson ImmunoResearch Europe Ltd., Suffolk, United Kingdom) for 1 h 30 min at 37 °C in blocking solution. Labelling of nucleus was performed at the end of immunolabelling protocol using DAPI (1:5000, Sigma–Aldrich) diluted in PBS for 15 min at 22 °C. After embedding in Fluoroprep (bioMERIEUX Benelux S.A./N.V., Brussels, Belgium), coverslips were then examined with a fluorescent inverted microscope (Evos fl, AMG, Westburg, Leusden, Belgium).

3. Results

3.1. Characterization of astrocytes morphology

Primary cultures of astrocytes maintained in culture medium containing 3% FBS were treated during 3 or 7 days with TNF- α (20 ng/mL), dBcAMP (150 μ M) or the G5 supplement. Changes in cell morphology during the treatments were monitored after 3 and 7 days by phase contrast microscopy and immunocyto labelling of GFAP. At both 3 and 7 days (Fig. 1A and B), untreated cultures mainly contained polygonal cells with few or no processes (protoplasmic morphology). In cells treated with TNF- α , a decrease in GFAP immunostaining was already noticed after 3 days (Fig. 1A). After 7 days, these cultures mainly consisted of typically

triangular-shaped cells with off-centred nuclei (Fig. 1B). Hence, no marked change in astrocyte morphology was evidenced by phase contrast microscopy (not shown). At variance, as early as 3 days after initiating the treatment with dBcAMP or the G5 supplement, the cultures show marked changes in the cell morphology. Thus, these treatments promoted the extension of several processes and condensation of the cell bodies (fibrous morphology) (Fig. 1A and B). The intensity of GFAP staining within cellular processes was noticeably higher in astrocytes treated for 7 days with dBcAMP, as compared to cells exposed to the G5 supplement (Fig. 1B). Finally, these analyses revealed different cell densities in dBcAMP and G5 supplement-treated cells, confirming the respective decrease and increase in cellular proliferation as previously reported (Tilleux et al., 2009; Vermeiren et al., 2005). The GFAP expression was also examined in qPCR studies on RNA samples extracted from the different cultures, revealing that GFAP mRNA appeared considerably decreased in cells exposed to TNF- α for 3 days (Fig. 1C) and this decrease persisted at day 7 (Fig. 1D). A modest increase in GFAP expression was evidenced in cells exposed to dBcAMP (significant at day 7) while no significant influence of the G5 supplement was observed. This latter observation suggests that intense GFAP staining results from condensation of the cytosol.

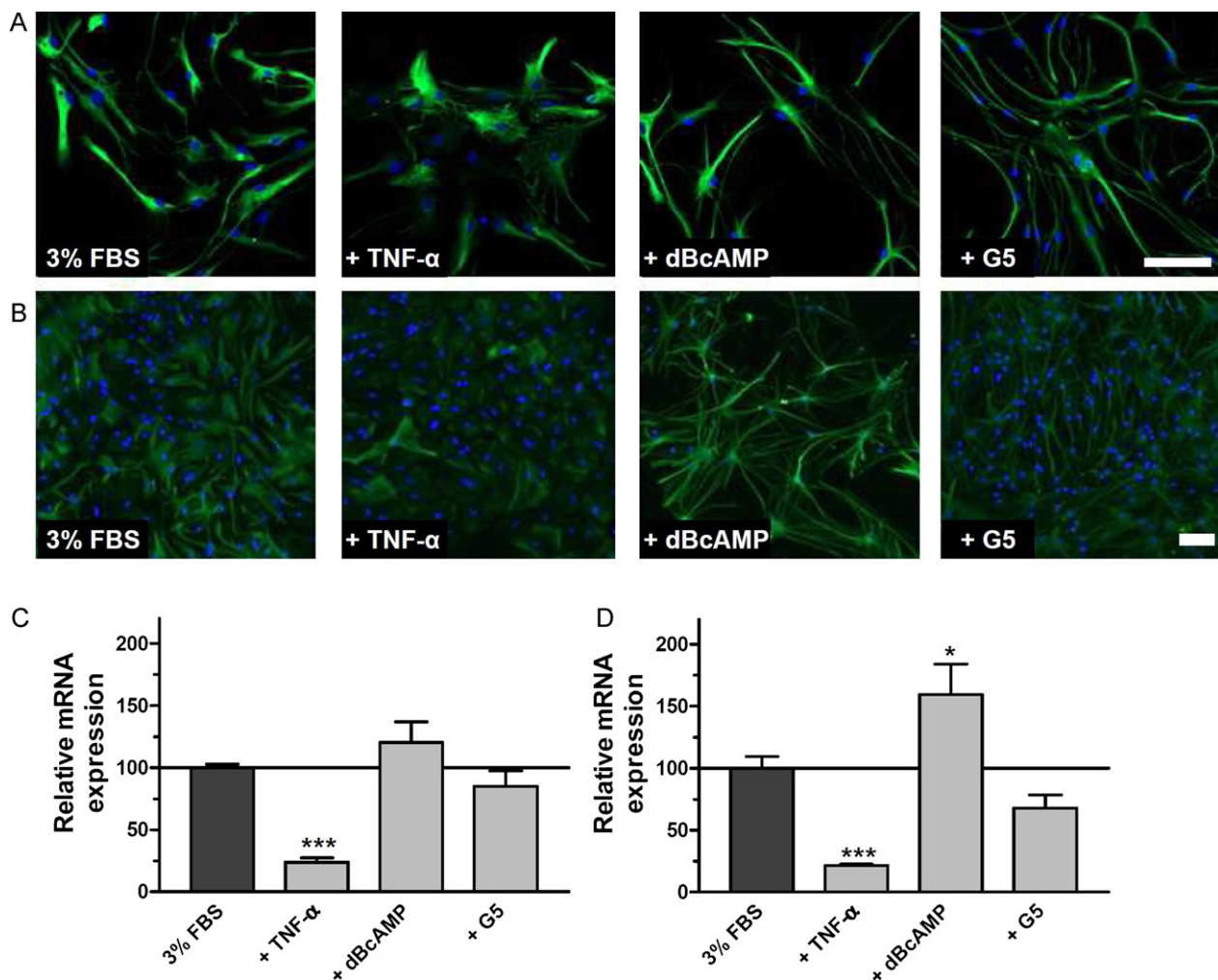


Fig. 1. Astrocytes treated with TNF- α , dBcAMP or the G5 supplement show altered morphology and GFAP expression. Astrocyte cultures were exposed to TNF- α (20 ng/mL), dBcAMP (150 μ M) or the G5 supplement. The cell morphology was examined by immunocyto labelling of GFAP after 3 (a) or 7 days (b). Shown are representative pictures from three independent experiments (scale bars = 100 μ m). The relative mRNA expression of GFAP after 3 (c) or 7 days (d) of treatment was measured by qPCR and expressed in percentage of the signal measured in control conditions (3% FBS). Data shown represent the mean with SEM from five independent experiments performed in duplicate and were compared using one-way ANOVA followed by the Dunnett's post hoc test for multiple comparisons (* p < 0.05, *** p < 0.001).

3.2. TNF- α , dBcAMP and the G5 supplement upregulate both GLT-1a and GLT-1b mRNAs

Plasmid DNA constructs containing the GLT-1 isoform coding sequences were used to standardize the amplifications with specific primers, allowing absolute determination of mRNA copy number. After 3 days in control conditions (3% FBS), GLT-1a mRNA largely surpassed GLT-1b mRNA (Fig. 2). Cultures maintained for 7 days showed a substantial decline in GLT-1a mRNA ($p < 0.05$) while GLT-1b mRNA remained constant, indicating a differential constitutive expression of GLT-1a and GLT-1b in cultured astrocytes. These data suggest that despite culture medium renewal, *in vitro* ageing of the cells is associated with a decreased expression of GLT-1a.

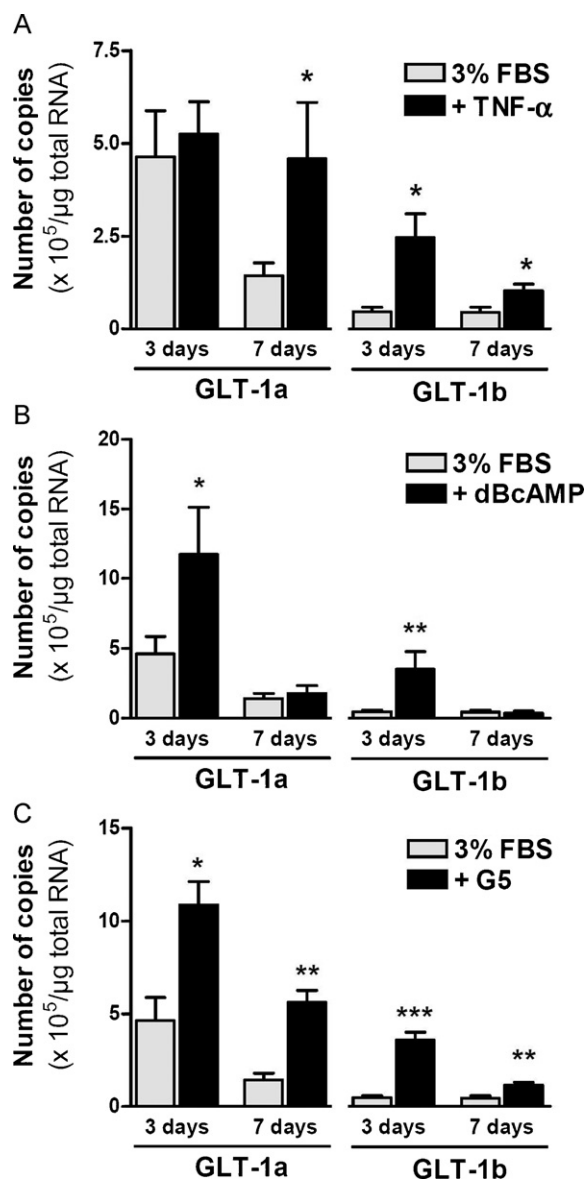


Fig. 2. Genetic expression of GLT-1a and GLT-1b isoforms in primary astrocytes. Absolute quantification of GLT-1a and GLT-1b mRNA was performed by qPCR using the corresponding cloned cDNA sequences as standards. The expression of both transcripts was examined in cells exposed to TNF- α (a), dBcAMP (b) and the G5 supplement (c) for 3 or 7 days. Data shown represent the mean with SEM from five independent experiments performed in duplicate and were compared by two-way ANOVA followed by the Bonferroni's post hoc test for multiple comparisons (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Exposure of cells to TNF- α significantly upregulated both GLT-1a and GLT-1b transcripts (Fig. 2A). However, such increase was only detected after 7 days of exposure for GLT-1a whereas a robust increase in GLT-1b mRNA was observed at day 3, evidencing a more pronounced and rapid influence of TNF- α on the expression of GLT-1b as compared to GLT-1a. Also, the addition of TNF- α appeared to balance the spontaneous time-dependent decrease in GLT-1a expression observed in control conditions. Both GLT-1a and GLT-1b mRNA were upregulated after exposure of the astrocytes to dBcAMP for 3 days, respectively by 2.5 and 7.5-fold (Fig. 2B). However, this effect was no longer observed for both variants after 7 days of treatment, indicating that the influence of dBcAMP on GLT-1 mRNAs expression was transient. Finally, the addition of the G5 supplement to the culture medium resulted in a 2- and 4-fold increased GLT-1a mRNA after 3 and 7 days, respectively (Fig. 2C). On the opposite, the upregulation of GLT-1b was stronger at 3 days (7.6-fold increase) as compared to 7 days of treatment (2.6-fold increase). All together, these results suggest that even though both GLT-1a and GLT-1b are influenced by the soluble factors tested, the two transcripts show distinct regulatory profiles in terms of kinetic and amplitude.

3.3. Differential regulation profile of GLT-1a and GLT-1b protein by soluble factors

To further characterize the influence of soluble factors on the expression of GLT-1a and GLT-1b at the protein level, we developed a specific monoclonal antibody against the C-terminus of the rat GLT-1b. The specificity of this original GLT-1b antibody, as well as the GLT-1a antibody available from Chemicon (Ref #AB1783) was validated using protein extracts from transiently transfected COS-7 cells expressing GLT-1a or GLT-1b along with cortex tissue from a Sprague–Dawley adult rat (Fig. 3). Antibodies raised against GLT-1b specifically recognized a band in cortex tissue samples at approximately 70 kDa, consistent with the expected molecular weight of GLT-1 (Fig. 3B). It also labelled three heavier bands at approximately 90, 130 and 165 kDa. The two upper bands likely correspond to multimers of GLT-1 as previously documented in neural cell cultures as well as in heterologous expression systems (Chen et al., 2002; Gonzalez-Gonzalez et al., 2009). Besides, the nature of the 90 kDa band remains so far unresolved. When tested with samples from transfected COS-7-GLT-1b cells, this antibody specifically labelled three bands of 80, 145 and 190 kDa. These bands were not detected in sample from untransfected cells (not shown) or from COS-7-GLT-1a cells. Of note, a 55 kDa band was systematically detected in COS-7 cells, which was confirmed as non-specific labelling by the secondary antibody (not shown). Besides, the commercially available GLT-1a antibody specifically detected a 70 kDa and a 105 kDa bands as well as a more diffuse band over 130 kDa in the sample from rat cortex (Fig. 3A). This diffuse labelling resolved into 140 and 190 kDa bands when reducing the detection time. Confirming the specificity of the signals, these immunoreactive bands were also observed in samples from COS-7-GLT-1a cells and not in samples from untransfected cells (not shown) or from COS-7-GLT-1b cells. Differences in the molecular weight of immunoreactive bands were systematically observed when comparing cortex samples and transfected cells. This was observed in all tested conditions of protein extraction (data not shown), suggesting distinct protein processings in native tissue and transfected cells.

Protein extracts from astrocytes maintained in the absence of added soluble factors were analyzed by Western-blotting using the GLT-1a and GLT-1b antibodies. Expression of both isoforms was substantially higher after 3 days in the 3% FBS containing medium as compared to 7 days old cultures (Fig. 4A). Thus, *in vitro* astrocytes ageing appears to decrease the expression of the GLT-1

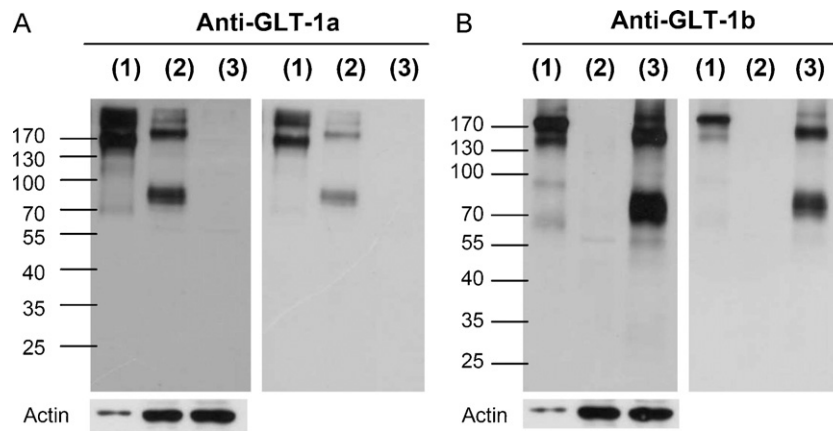


Fig. 3. Validation of the selective immunodetection of GLT-1 isoforms. Protein extracts from adult rat brain (2.5 μ g, lane 1) and transfected COS-7 cells expressing the recombinant GLT-1a (20 μ g, lane 2) or GLT-1b (20 μ g, lane 3) were immunoprobed with a commercially available anti-GLT-1a antibody (panel A) or with a newly designed monoclonal anti-GLT-1b antibody (panel B). In order to detect faint signals and to facilitate the analysis of intense immunoreactive bands, long and short exposures of each blot are shown (left and right, respectively).

transporters. In the presence of TNF- α , GLT-1a was increased by 2.2-fold after 3 days but returned to control levels after 7 days (Fig. 4B). In contrast, GLT-1b protein progressively increased in response to TNF- α , and was more than doubled after 7 days. Addition of dBcAMP robustly and time-dependently stimulated the expression of both proteins, reaching up to 10-fold the control level for each isoform after 7 days of exposure (Fig. 4C). Finally, astrocytes exposed to the G5 supplement for 3 or 7 days showed enhanced level of GLT-1 splice variants as compared to control conditions (respectively 6-fold and 12-fold increase in GLT-1a and GLT-1b immunoreactivity after 7 days of exposure) (Fig. 4D). All together, these results confirm that TNF- α , dBcAMP and the G5 supplement influence the protein expression of GLT-1a and GLT-1b. However, the two variants show distinct regulatory profiles and it is noteworthy that variations in the expression of the protein fail to specifically correlate with changes of the corresponding mRNA.

4. Discussion

Alternative splicing of pre-mRNA operates for more than 70% of multi-exon genes in the human genome (Johnson et al., 2003). Splicing affects gene expression by removing or inserting regulatory elements controlling translation, mRNA stability or localization. It also interferes with protein expression by exon skipping or intron inclusion, promoting the expression of alternative spliced isoforms. Additionally, the modifications of mRNA coding sequences affect the subcellular localization, activity or stability of encoded protein isoforms. Even though the physiological implication of GLT-1 alternative splicing is not established, some key evidence highlights the importance of C-terminal variants. Indeed, it has been shown that GLT-1b, but not GLT-1a, can interact with PDZ proteins, conferring increased stability of GLT-1b at the cell surface (PSD-95 interaction) or reducing its PKC α -induced turnover (PICK1 interaction) (Bassan et al., 2008; Gonzalez-Gonzalez et al., 2008, 2009). Nonetheless, GLT-1 is expressed as trimers on the cell surface (Yernool et al., 2004) and GLT-1 splice variants tend to co-immunoprecipitate from brain samples, confirming their interaction in native tissues (Bassan et al., 2008; Gonzalez-Gonzalez et al., 2008, 2009). Hence, it is proposed that through PDZ protein-mediated trafficking or anchoring, the GLT-1b variant could indirectly influence the entire GLT-1 transporter complex.

In several neurological disorders, the expression of GLT-1 isoforms appears to be differentially regulated suggesting that mechanisms underlying splicing of this transporter can be

modulated (Goursaud et al., 2009; Maragakis et al., 2004; Scott et al., 2010). Using a newly designed and validated anti-GLT-1b specific monoclonal antibody as well as optimized PCR conditions enabling quantification of mRNA copy numbers, this study examined the differential regulation of two C-terminal GLT-1 splice variants in astrocyte cultures in response to different stimuli. In addition to dBcAMP and the G5 supplement, commonly used to promote the maturation/differentiation of astrocytes and known to enhance GLT-1a expression in primary cultures (Chen et al., 2002; Tilleux et al., 2009; Vermeiren et al., 2005), our interest was focused on the pro-inflammatory cytokine TNF- α . In agreement with data from the literature, TNF- α , dBcAMP and the G5 supplement were found to deeply alter astrocyte morphology and proliferation, validating our model and culture conditions. Compared to naive/immature cultures, mainly consisting in typical polygonal protoplasmic astrocytes, decreased GFAP mRNA and GFAP immunoreactivity were observed in TNF- α -treated astrocytes which adopted a triangular-morphology. Besides, even though dBcAMP and the G5 supplement show opposite influences on cell proliferation, they both promote the growth of cell processes leading to the so-called stellate morphology.

First focusing on astrocytes maintained in standard conditions, we show that both GLT-1a and GLT-1b proteins tend to decrease during the maintenance of the culture. Previous studies have shown that in long-term cultures, astrocytes exhibit hypertrophy, increased GFAP expression and higher basal glutamate uptake but appear more sensitive to oxidative stress, suggesting that *in vitro*, astrocytes develop senescence characteristics and increase their glutamate transport capacity. Nonetheless, this increased glutamate handling, regarded as a compensatory mechanism, mainly involves the glutamate aspartate transporter (GLAST) (Pertusa et al., 2007), and suggests that subtype-specific regulations of glutamate transporters operate during astrocytes ageing *in vitro*.

The influence of TNF- α on glutamate transporter expression and function has been examined in several studies with rather inconsistent results likely reflecting the diversity of models and experimental conditions tested (Boycott et al., 2008; Liao and Chen, 2001; Romera et al., 2004; Zou and Crews, 2005). We have previously demonstrated that soluble mediators released by LPS-induced microglial activation lead to an upregulation of GLT-1 mRNA and activity in primary cultured astrocytes, an effect that is recapitulated by direct exposure of primary astrocytes to TNF- α (Tilleux et al., 2009). Consistent with this study examining total GLT-1 expression, we herein demonstrate that this cytokine

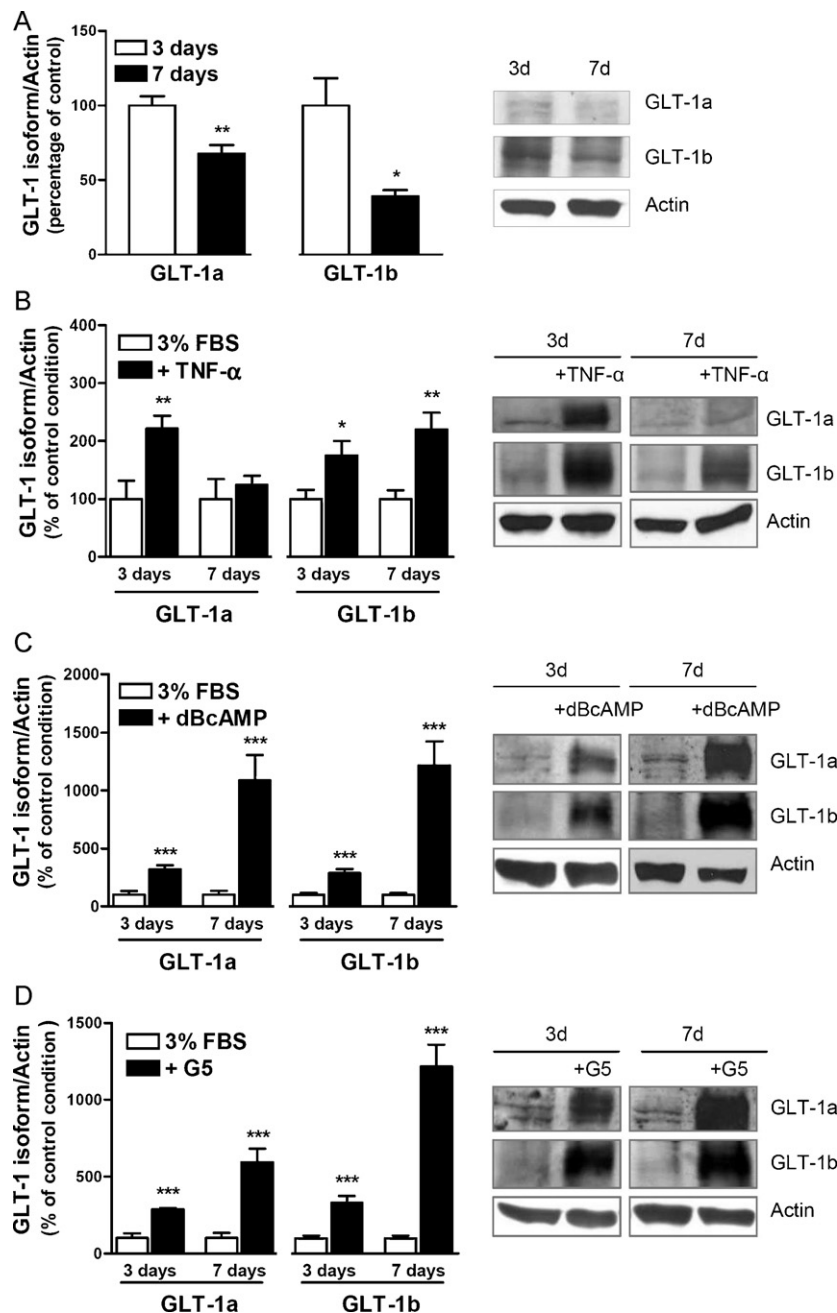


Fig. 4. Proteic expression of GLT-1a and GLT-1b isoforms in primary astrocytes. Using a commercially available anti-GLT-1a antibody and the newly designed monoclonal rat anti-GLT-1b antibody, the expression of the two splice variants was examined in cells maintained in culture for 3 or 7 days (A). The expression of isoforms was further analyzed in astrocytes exposed to TNF- α (B), dBcAMP (C) and the G5 supplement (D) for 3 or 7 days. Immunoblots shown are representative of eight independent experiments. Graphs represent quantitative data from densitometric analyses of GLT-1a or GLT-1b immunoreactive signals normalized to actin and data are expressed in percent of the signal detected for cells maintained for 3 days in control condition (a) or for the corresponding control cultures (b)–(d). Data were compared using the two-tailed Student's *t*-test (* p < 0.05, ** p < 0.01, *** p < 0.001).

induces the expression of both GLT-1a and GLT-1b *in vitro*. Similarly, dBcAMP and the G5 supplement strongly promoted both isoforms, with however discrepancies that were noticed when examining the regulation of mRNA or protein expression. Obviously, such differences could reflect distinct kinetics of mRNA processing and translation. Indeed, for both isoforms, the mRNA inductions were essentially observed after only 3 days of treatment whereas protein increases are maintained at later time points. Furthermore, both GLT-1a and GLT-1b appear to be similarly regulated by dBcAMP or the G5 supplement, even though dBcAMP transiently promotes GLT-1 mRNA expression while the G5 supplement continuously stimulates this mechanism. With

respect to TNF- α , we noticed a continuous upregulation of GLT-1b mRNA and protein, while this cytokine mediated a multifaceted influence on GLT-1a. Thus at variance with immunoblotting studies showing a transient increase in GLT-1a protein in response to TNF- α , a sustained induction of the corresponding mRNA was observed by absolute qPCR. These observations suggest that several and sometimes opposite mechanisms could support the TNF- α -induced regulation of GLT-1 isoforms. In fact, the control of ribosomal machinery recruitment, the presence of untranslated mRNA species and the individual half-time of proteins are amongst the main mechanisms influencing the protein/mRNA ratio (Maier et al., 2009; de Sousa et al., 2009). Hence, upregulation of EAAT2b

protein has been evidenced in the brain of ALS patients while the expression of the EAAT2a is decreased (Maragakis et al., 2004). Also, we recently demonstrated an opposite regulation of GLT-1a and GLT-1b in a transgenic rat model of ALS (Goursaud et al., 2009). Primary cultures of astrocytes isolated from mice carrying the same ALS related mutation of hSOD1 have been shown to express high levels of several inflammatory cytokines, including TNF- α (Hensley et al., 2006). It is tempting to suggest that TNF- α participates in the altered balanced expression of GLT-1 splice variants. Of note, TNF- α has been shown to promote caspase-3 activation which cleaves and inactivates EAAT2 (Boston-Howes et al., 2006). Using rat brain samples, we have recently accumulated evidence indicating that caspase-3-mediated inactivation primarily concerns GLT-1a whereas GLT-1b is not affected (Goursaud and Hermans, unpublished data). Nonetheless, the mechanisms that would increase GLT-1a protein without changes in the corresponding mRNA remain unclear. Physiological controls of the transporter stability have not been reported but recent studies have identified several compounds that specifically increase the translation of EAAT2 transcripts, usually silenced by long 5' UTRs (Colton et al., 2010). Moreover, with one of the longest 3' UTRs of the human genome, EAAT2 mRNA contains different domains that can enhance or reduce mRNA translation (Bette et al., 2010; Kim et al., 2003). In conclusion, we have herein shown that while dBcAMP and the G5 supplement upregulate both C-terminally splice variants of GLT-1 in primary cultures of astrocytes at the mRNA and protein levels, the pro-inflammatory cytokine TNF- α exhibits a dual influence on GLT-1a/GLT-1b expression. Indeed, as GLT-1b protein show sustained upregulation, the GLT-1a is transiently induced. Conversely, both GLT-1 splice variants are increased at the mRNA level. Further work should determine the mechanisms underlying TNF- α -regulated GLT-1 expression, notably the involvement of caspase-3-associated signalling pathways. Besides, the present study demonstrates that the alternative splicing of GLT-1 or the stability of the splice variant mRNAs can be regulated. These data certainly confirm the need and relevance of further studies concerning the functions of each GLT-1 isoforms, which should shed light on the regulation of glutamate homeostasis in the brain and in neurological disorders where glutamate uptake is disrupted.

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