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Thiamine deficiency results in release of soluble factors that disrupt mitochondrial membrane potential and downregulate the glutamate transporter splice-variant GLT-1b in cultured astrocytes

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ABSTRACT

Loss of astrocytic glutamate transporters is a major feature of both thiamine deficiency (TD) and Wernicke's encephalopathy. However, the underlying basis of this process is not well understood. In the present study we have investigated the possibility of release of astrocytic soluble factors that might be involved in the regulation of the glutamate transporter GLT-1b in these cells. Treatment of naïve astrocytes with conditioned media from astrocytes exposed to TD conditions resulted in a progressive decrease in glutamate uptake over 24 h. Immunoblotting and flow cytometry measurements indicated this was accompanied by a 20–40% loss of GLT-1b. Astrocytes exposed to either TD or TD conditioned media showed increased disruption of mitochondrial membrane potential compared to control cells, and treatment of astrocytes with TD resulted in an increase in the pro-inflammatory cytokine TNF-α and elevated levels of phospho-IκB fragment, indicative of increased activation of NF-κB. Inhibition of TNF-α activity with the use of a neutralizing antibody blocked the increased NF-κB activation, while inhibition of NF-κB ameliorated the decrease in GLT-1b and reversed the decrease in glutamate uptake occurring with TD treatment. Together, these findings indicate that astrocytes exposed to TD conditions show responses suggesting that soluble factors released by these cells under conditions of TD play a regulatory role in terms of glutamate transport function and mitochondrial integrity.

1. Introduction

Thiamine deficiency (TD), the cause of Wernicke's encephalopathy (WE), results in histologic lesions in focal areas of the brain that include the thalamus, inferior colliculus, and brainstem structures such as the vestibular nuclei and inferior olives. The underlying basis of this damage, despite decades of investigation, is unresolved. During the development of these lesions, both neurons and astrocytes are affected, with neuronal cell loss being a consequence of the deficiency. Astrocytes are highly abundant in the brain and play an important role in normal synaptic transmission [1,2]. Many functions of astrocytes are important in determining the tissue response to TD, including their ability to take up glutamate and reduce excitotoxicity.

TD leads to inhibition of oxidative decarboxylation of pyruvate and α-ketoglutarate, leading to decreased ATP production in focal regions of the brain [3]. During the development of TD, both neuronal and astrocytic intermediary metabolism are impaired and protective astrocytic functions such as glutamate uptake are compromised [4].

Treatment with pyrithiamine, a thiamine antagonist, results in downregulation of the astrocytic glutamate transporters GLT-1 and GLAST [5], a process also occurring in human cases of WE [6]. Although the mechanism of this transporter dysfunction is still unclear, studies have reported a role for extrinsic factors in the regulation of glutamate transporter activity. For example, elevated production of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) is known to induce excitotoxicity by inhibition of glutamate uptake [7]. In the present study, we therefore investigated whether soluble factors such as an inflammatory TNF-α-based process may be involved in TD-induced loss of glutamate transporter activity.

2. Materials and methods

2.1. Cell culture preparation

Astrocyte cultures from newborn rats were prepared as previously described [8]. At confluency, astrocytes were maintained in

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DMEM containing 10% horse serum and treated with 0.1 mM dibutyryl cyclic AMP (dBcAMP) continuously thereafter. To induce TD, cells were exposed to a custom-designed DMEM media lacking in thiamine (Invitrogen, Burlington, ON) and containing 5% horse serum, in the presence of the thiamine antagonist pyrithiamine (10 μM). Control astrocytes were treated with TD media in which normal levels of thiamine (4 mg/L) had been added. Media was changed every 3–4 days. At least 95% of cells were determined to be astrocytes based on GFAP immunocytochemistry. Conditioned media was collected from astrocytes exposed to TD conditions for 10 days in which the media was earlier changed after 4 and 7 days of exposure to TD to restrict the accumulation of toxic metabolites in the media. Naïve astrocytes were then exposed to this conditioned media for up to 24 h.

2.2. Production of GLT-1b antibody

A polyclonal antibody against the synthetic peptide CKVPFPFL-DIET corresponding to the last 12 amino acids (amino acid 549–560) of the GLT-1b splice-variant [9] and conjugated to keyhole limpet hemocyanin (Sheldon Biotechnology Centre, Montreal, QC) was generated in rabbits using a standard protocol (Comparative Medicine & Animal Resources Centre, McGill University, Montreal, QC). The GLT-1b sequence that was chosen represents a unique carboxyl terminal region and has no similarity with other sequences in the standard gene database. The specificity of the antiserum was initially evaluated by immunoblotting as previously described [10]. Immunodetection was performed using the primary antiserum at a range of dilutions (0.01–0.1 μg/ml).

2.3. Immunoblotting studies

Experiments involving immunoblotting were performed as previously described [10]. Following protein transfer, membranes were incubated with rabbit polyclonal antisera directed against GLT-1b or phosphor IκB (Cell Signaling Technology, Inc., Danvers, MA). Reblocking was followed by incubation with horseradish peroxidase-coupled anti-rabbit IgG (0.01 lg/ml) secondary antiserum. Each incubation step was of 1 h duration following which blots were washed several times with buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween-20). For the detection of specific antibody binding, the membranes were treated in accordance with ECL-kit instructions and developed on photosensitive X-OMAT film. Signal intensities were subsequently measured by densitometry using Image J software (National Institutes of Health, Bethesda, MD). Linearity of the relationship between optical density and protein concentration was verified using appropriate standard curves.

2.4. Immunocytochemistry

Immunocytochemistry was perfomed as previously described [5]. Briefly, astrocytes were washed in PBS and fixed for 10 min with 10% neutral buffered formalin. After washing cells were blocked for 30 min with 1% donkey serum in PBS, and then incubated with 1% donkey serum and polyclonal goat antisera directed against GFAP (1:200, Santa Cruz Biotechnology, Santa Cruz, CA). Sections were then washed (10 min) and incubated for 1 h with Alexa Fluor-488 (green) secondary antibody (1:200), then mounted in Prolong Gold AntiFade reagent and examined using an Olympus BX51 microscope and attached Spot RT digital camera. Negative controls consisted of omission of primary or secondary antibody, resulting in loss of immunoreactivity. Images were processed using Image-Pro Plus 6.2 image analysis software (Media Cybernetics, Inc., Bethesda, MD).

2.5. Changes in mitochondrial membrane potential

Cultured astrocytes were analyzed for changes in the mitochondrial membrane potential ($\Delta\psi m$) using the membrane potential-dependent aggregate-forming lipophilic cation JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide) (Invitrogen, Burlington, ON). Cells were assayed according to the manufacturer's instructions. Changes in astrocytic $\Delta\psi m$ in TD and TD media treated astrocytes were quantified by FACScan flow cytometry. Images were captured using an Olympus BX51 microscope (original magnification ×100) and merged using Image J software.

2.6. Flow cytometry

Astrocytes were harvested in papain solution (Worthington Biochemical Corp., Lakewood, NJ) for 15–20 min and resuspended in flow cytometry buffer, consisting of 1% fetal bovine serum (Invitrogen, Burlington, ON) in 1× PBS, pH 7.2. Cells were counted and diluted to a density of 1×10^6 cells/ml of buffer. Aliquots of 1×10^5 cell were incubated with polyclonal antisera directed against GLT-1b (Cell Signalling Technology, Inc., Danvers, MA) and polyclonal goat antisera directed against GFAP (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), for 30 min on ice. Cells were washed in buffer, and then JC-1 or specific secondary fluorescent-conjugated antibodies, Alexa Fluor-592 (red) conjugated anti-mouse IgG and Alexa Fluor-488 (green) conjugated anti-rabbit IgG (Invitrogen, Burlington, ON) were added at the appropriate dilution (1:100) and incubated on ice for 15 min. Viable cells were gated by light scatter and were analyzed by FACScan (BD LSRII) (BD Biosciences, Mississauga, ON) using a Cell Quest program (BD Biosciences, Mississauga, ON). Fluorescence background was measured using unlabeled cells and cells labeled with secondary antibody alone; these set gating parameters between positive and negative cell populations. Cell aggregates and small debris were excluded from analysis or isolation on the basis of side scatter (measuring cell granularity) and forward scatter (measuring cell size). Fluorescent intensities for cells in the population were plotted as quadrants and/or histograms using CellQuest software (BD Biosciences, Mississauga, ON).

2.7. Glutamate uptake

Glutamate uptake was performed using [^{3}H]-D-aspartate as previously described [8,10]. Briefly, cells were incubated in DMEM media containing D-aspartate, a non-metabolized glutamate analog, and 0.2 μCi/ml of [^{3}H]-D-aspartate in 5% CO_2/95% air at 37 °C, the incubation time for all experiments being of 2 min duration. Uptake was stopped by aspiration of the media and rapid washing of cells three times with ice-cold PBS. Cells were then harvested in 0.5 ml of 1 M NaOH. Sample aliquots were measured for protein content and the radioactivity for determination of the uptake rate was measured by liquid scintillation counting.

2.8. Measurement of TNF-α using ELISA and treatment of astrocytes with anti-TNF-α neutralizing antibody

Protein levels of TNF-α were measured in astrocyte cultures using a commercial ELISA kit (R&D Systems, Minneapolis, MN; Invitrogen, Burlington, ON) according to the manufacturer's instructions. The plates were read at 450 nm and the absorbances were converted to pg/ml using standard curves prepared with recombinant cytokines. Astrocytes were treated with an anti-TNF-α neutralizing antibody (R&D systems, Minneapolis, MN) at a concentration of 0.5 μg/ml according to the manufacturer's guidelines.

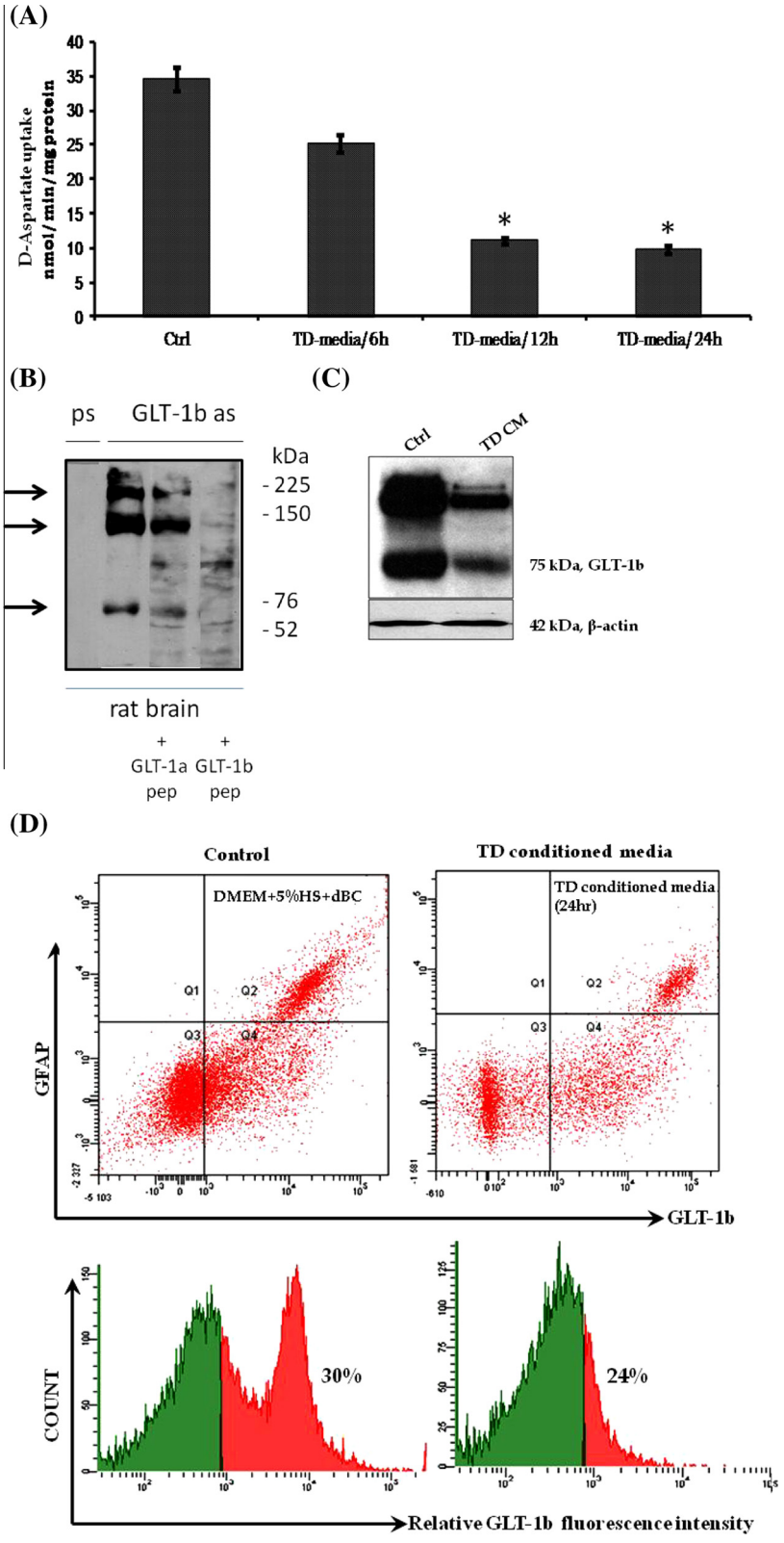

Fig. 1. Effects of TD conditioned media treatment on glutamate transport function in naïve astrocytes. (A) Glutamate uptake. Cells were exposed to TD conditioned media for different times (6 h, 12 h, 24 h) and uptake was measured at a D-aspartate concentration of 50 μM. (B) Immunoblot characterization of GLT-1b antiserum. Proteins from adult rat brain tissue (30 μg) were analyzed by immunoblotting using anti-GLT-1b antiserum (as). Pre-serum (ps) (from rabbits prior to GLT-1b peptide immunization showed no immunoreactivity to brain proteins. Anti-GLT-1b recognized major bands at 66, 130, and 200 kDa (arrows). This immunoreactivity was abolished when the antiserum was preabsorbed with GLT-1b peptide (pep) antigen. In addition, anti-GLT-1b antiserum did not cross-react with GLT-1a peptide antigen. (C) Effect of TD conditioned media on GLT-1b levels in naïve astrocytes. Cells exposed to conditioned media (CM) showed loss of GLT-1b with immunoblotting. (D) Flow cytometry analysis of GLT-1b levels. Upper panels show the two parameter cell distribution histograms for control and TD conditioned media groups with GFAP (*y*-axis) and GLT-1b (*x*-axis). The lower panels show the relative fluorescence intensity for GLT-1b in the total cell population. Actual fluorescence intensities for GLT-1b are indicated as percent values shown and determined from the Q4 quadrant. Conditioned media was obtained from astrocytes exposed to thiamine-deficient conditions that included a lack of thiamine and presence of pyrithiamine (10 μM) for 10 days. All cultures were harvested on the same day for each experiment. Results are expressed as ±S.E.M. of six to nine separate determinations. $^{*}p < 0.05$ compared to controls as determined by one-way ANOVA with post hoc Dunnett's test for multiple comparisons.

2.9. Statistical analysis

Statistical analysis was performed using either the Mann–Whitney *U*-test or one-way ANOVA with post hoc testing for multiple comparisons. A probability of $p < 0.05$ was chosen to establish significance between groups. Data were analyzed using Prism 4.0 (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Effects of TD conditioned media on glutamate transport in astrocytes

To examine if astrocytes respond to conditioned media from TD treated cells, we studied its effect on glutamate uptake and release in naïve astrocytes exposed to conditioned media for 24 h. Fig. 1A shows a decrease in glutamate uptake with time with uptake reduced by over 70% after 24 h. Next, we assessed whether these alterations in glutamate transport were caused by a change in the regulation of GLT-1b using immunoblotting. We produced a polyclonal rabbit-derived GLT-1b antibody against a peptide synthesized using the published sequence of GLT-1b. When used in immunoblotting of rat brain tissue, the GLT-1b antiserum produced major bands at 66 kDa, 130 kDa and 200 kDa (Fig. 1B). These bands represent the monomer, dimer and trimer forms of the protein. The specificity of the antiserum was tested by inclusion of the peptide against which the antibody was directed. The GLT-1b peptide blocked the appearance of all three bands (Fig. 1B). On the other hand, inclusion of the GLT-1a peptide antigen showed no major cross-reactivity with this splice-variant (Fig. 1B). Further examination of the specificity of the GLT-1b antiserum by immunoblotting of primary cultures of astrocytes with no prior dBcAMP treatment, and which therefore do not express significant levels of GLT-1 protein failed to produce any monomer and multimer bands for this transporter splice variant (data not shown). Immunoblot analysis of conditioned media-treated naïve astrocytes indicated a 40% loss of GLT-1b compared to controls (Fig. 1C). To further examine this effect we used flow cytometric analysis, with 24 h treatment with TD conditioned media also showing a decrease in the level of GLT-1b (from 30% to 24% of the cell population) (Fig. 1D), consistent with the immunoblotting results given its limitations. Together, these findings indicate that soluble factors released from astrocytes exposed to TD conditions downregulate glutamate transporter levels and alter glutamate transport function.

3.2. Effect of TD conditioned media on mitochondrial function

We used JC-1 to probe mitochondrial function by imaging the monomeric form (green) and the aggregate form (red) of this fluorescent probe. An abrupt decrease in red fluorescence and an increase in green fluorescence in astrocytes exposed to TD

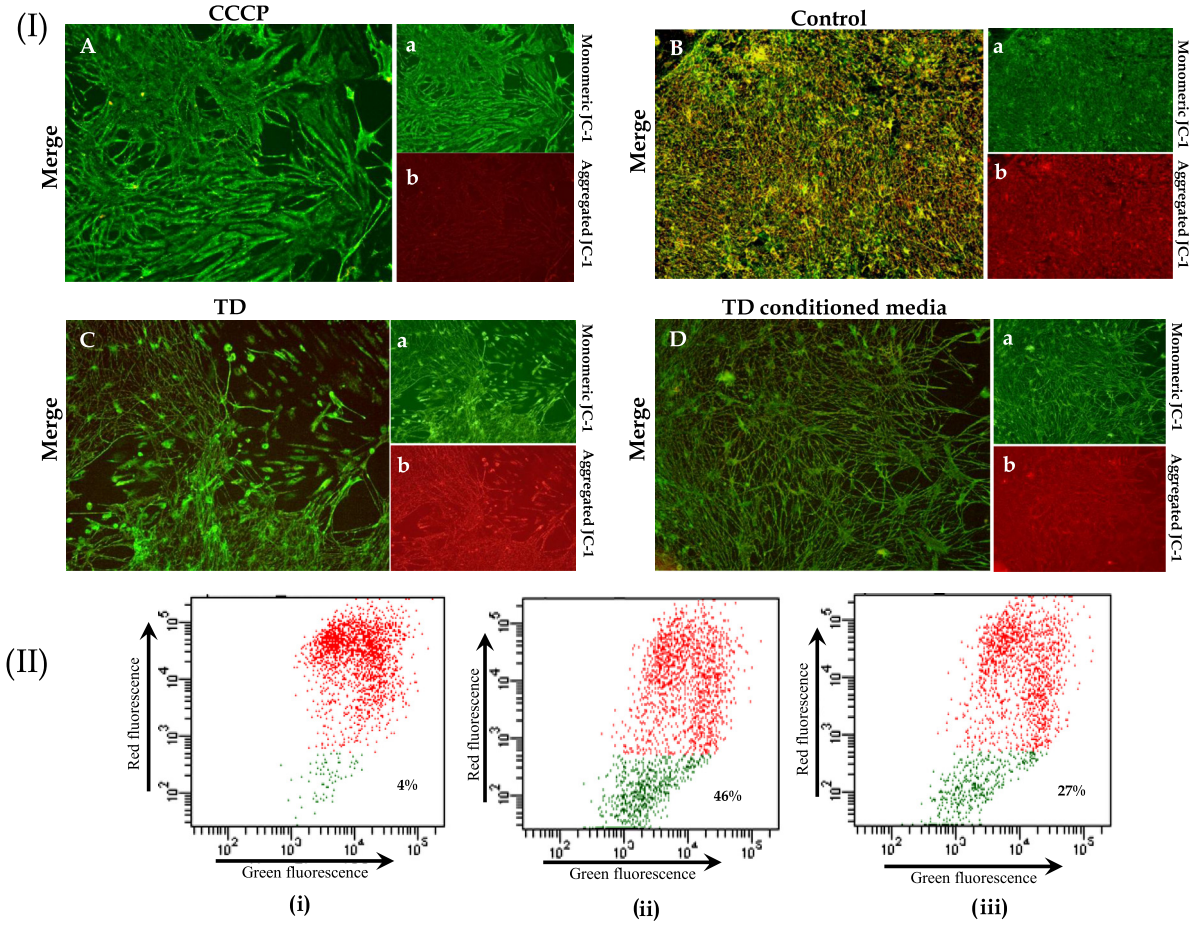

Fig. 2. Analysis of mitochondrial membrane potential ($\Delta\psi m$) in astrocytes with TD or after treatment of naïve astrocytes with TD conditioned media. Red fluorescence indicates JC-1 aggregates representing mitochondria with intact membrane potential whereas green fluorescence indicates JC-1 monomers representing de-energized mitochondria. (I) Images show treatment of (A) naïve astrocytes treated with the mitochondrial membrane potential disrupter CCCP, (B) control (naïve) astrocytes, (C) naïve astrocytes treated with TD, and (D) naïve astrocytes treated with TD conditioned media. (II) Flow cytometric determination of $\Delta\psi m$ changes in control astrocytes (i), astrocytes exposed to TD (ii), and naïve astrocytes treated with TD conditioned media (iii). Values represent mean intensity of JC-1 (green fluorescence) for 6 independent determinations in each group.

conditions were observed compared to control plates, indicating $\Delta\psi m$ collapse (Fig. 2I). Exposure of naïve astrocyte cultures to TD conditioned media for 24 h resulted in increased mitochondrial depolarization. Treatment with the mitochondrial membrane potential uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 1 μM/ml) for 10 min was used as a positive control in these experiments. Next, to quantitatively assay JC-1 signals, we used flow cytometry to compare the ratio of the monomeric form to that of the aggregate form (Fig. 2II). Accumulative fluorescence intensity for JC-1 monomer representing the extent of loss of $\Delta\psi m$ was significantly higher in TD (~46%) and TD conditioned media treated astrocytes (~27%) relative to control astrocytes (~4%).

3.3. Involvement of TNF-α in the loss of $\Delta\psi m$ in TD

To investigate the role of TNF-α and its associated NF-κB activation in $\Delta\psi m$ under conditions of TD, we first used an ELISA approach to specifically measure the relative levels of TNF-α in astrocytes exposed to TD conditions. Exposure of astrocytes to TD conditions resulted in large increases in the levels of TNF-α in the media over 10 days (Fig. 3I). Treatment of naïve astrocytes with TD conditioned media resulted in a substantial decrease in $\Delta\psi m$ (i.e. increased mitochondrial depolarization) compared to controls over the same time period (Fig. 3IIA and B). On the other hand, cells co-treated with TD conditioned media and a specific anti-TNF-α neutralizing antibody showed evidence of an amelioration of this decrease in $\Delta\psi m$ (3IIC). When naïve astrocytes were exposed to recombinant TNF-α (2 ng/ml), a large increase in mitochondrial depolarization occurred, not dissimilar to that observed with TD conditioned media (Fig. 3IID).

3.4. Involvement of TNF-α dependent NF-κB activation in glutamate transporter dysfunction

To investigate the role of TNF-α and its associated NF-κB activation in the loss of glutamate transport under conditions of TD, we first used immunoblotting to measure relative levels of the phospho-IκB fragment, indicative of increased activation of NF-κB in astrocytes exposed to TD conditions. TD treatment in astrocytes resulted in a large elevation in levels of phospho-IκB, while inhibition of TNF-α in astrocytes with TD by treatment with anti-TNF-α neutralizing antibody prevented the increase in phospho-IκB (Fig. 4A). In addition, exposure to the specific NF-κB inhibitor PDTC (100 μM) produced an amelioration of the loss of GLT-1b in astrocytes with TD (Fig. 4B) and restored glutamate uptake capacity in these cells (Fig. 4C).

4. Discussion

Excitatory amino acid transporters located on astrocytes play a crucial role in maintaining normal levels of extracellular glutamate.

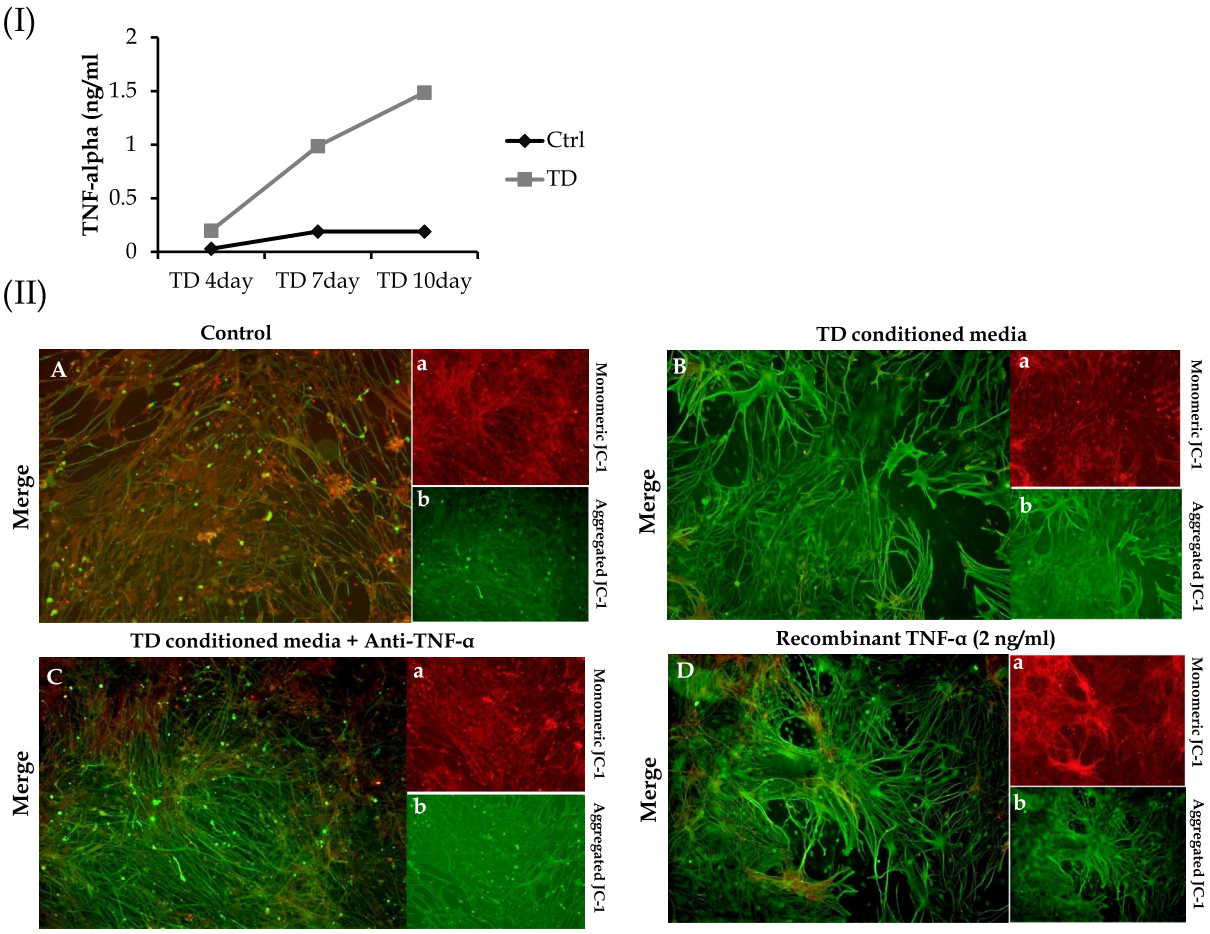

Fig. 3. Effects of TNF-α on mitochondrial membrane potential ($\Delta\psi m$) in astrocytes. (I) TD results in increased production of TNF-α in the astrocytes. (II) Images show (A) naïve astrocytes, (B) control (naïve) astrocytes treated with TD conditioned media (from astrocytes exposed to 10 days of TD, (C) naïve astrocytes treated with TD conditioned media and anti-TNF-α, (D) naïve astrocytes treated with exogenous recombinant TNF-α (2 ng/ml). Treatment of control (naïve) astrocytes with TD conditioned media altered mitochondrial membrane potential. Red fluorescence indicates JC-1 aggregates (a) representing mitochondria with intact membrane potential whereas green fluorescence indicates JC-1 monomers (b) representing de-energized mitochondria.

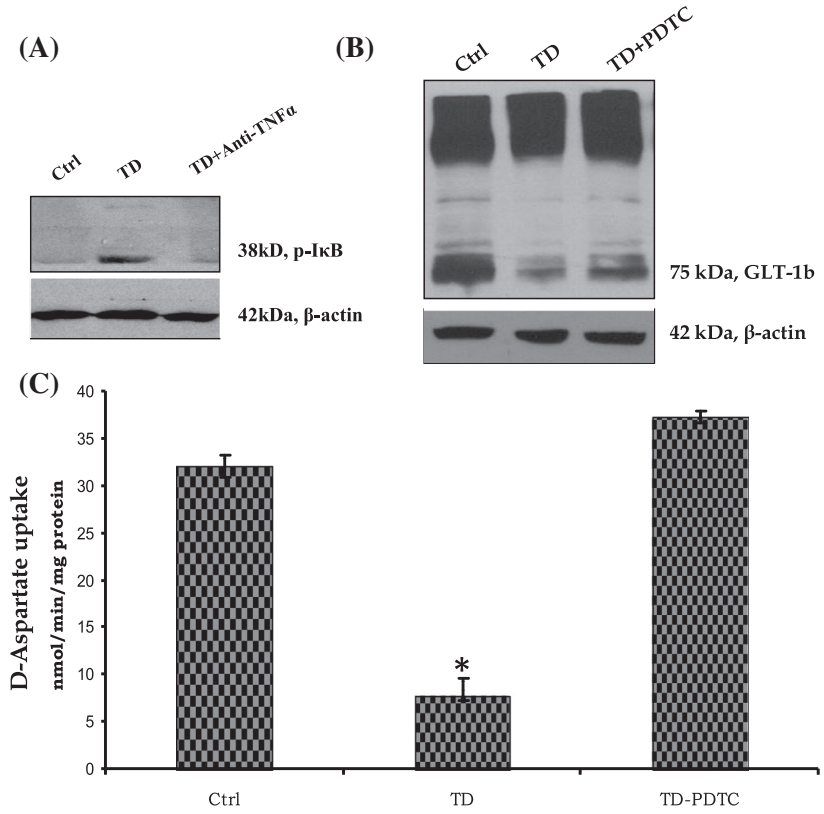

Fig. 4. Involvement of TNF-α dependent NFκB activation in glutamate transport dysfunction. (A) TD treatment resulted in increased levels of phospho-IκB (indicative of activation of NFκB), and inhibition of TNF-α using an anti-TNF-α neutralizing antibody blocked the heightened activation of NFκB. (B) Inhibition of NFκB using the specific NFκB inhibitor PDTC (100 μM) ameliorated the loss of GLT-1b in TD astrocytes, and (C) restored glutamate uptake capability in TD astrocytes. Results are expressed as ±S.E.M. of 6 independent determinations. $^{*}p < 0.05$ compared to controls as determined by one-way ANOVA with post hoc Dunnett's test for multiple comparisons.

Loss of these transporters in TD has been reported by us previously [5,10] and in human cases of Wernicke's encephalopathy [6]. However, the mechanism of the loss of transporters during TD is unclear. Previous studies have identified that soluble factors released by neurons can influence levels of astrocytic glutamate transporters [11,12]. Under conditions of impaired energy metabolism, astrocytes may produce chemical factors that not only can regulate glutamate transporter function from inside the cell, but these factors may also exit the cells and can potentially be measured and identified by their influence(s) on normal cells. In the present study, our results indicate that naïve astrocytes treated with culture media from astrocytes exposed to TD show a number of responses suggesting that soluble factors released by these cells under conditions of TD play a regulatory role in terms of glutamate transport function and mitochondrial integrity.

Treatment of normal astrocytes with TD conditioned media resulted in a loss of glutamate uptake in these cells. This indicates that soluble factors in the media previously exposed to astrocytes with TD exert a regulatory influence on glutamate transporters in these cells. Our findings that the decreased glutamate uptake with TD conditioned media is associated with a decrease in the levels of GLT-1b in naïve astrocytes indicate that soluble factors are able to alter protein levels of this transporter. Exactly how this regulation occurs is unclear.

To further examine the involvement of possible soluble factors in the regulation of glutamate transport function, an evaluation of their influence on mitochondrial functional integrity was performed. Impaired energy metabolism and mitochondrial dysfunction have been implicated in the pathogenesis of TD [3,13]. We therefore examined the issue of whether extracellular factors released by the astrocytes during TD have the ability to induce mitochondrial dysfunction using conditioned media from these TD treated cells and an imaging approach. Utilizing JC-1, our findings indicated that factors present in the TD conditioned media are able to produce depolarization of $\Delta\psi m$ within 24 h following exposure of the media to cells. Additional data obtained from flow cytometry confirmed the results of cellular imaging indicating that soluble factors released from TD treated astrocytes produce mitochondrial dysfunction in naïve astrocytes. Therefore, these soluble factors rapidly exert an effect on mitochondrial integrity, possibly leading to loss of GLT-1b due to the consequential negative effects on ATP production. Although naïve astrocytes were exposed to conditioned media containing thiamine-deficient conditions for up to 24 h, previous studies from our group have demonstrated that 4 day TD treatment is required for the activity of thiamine-dependent enzymes to be compromised in these cells and 7 days for loss of glutamate uptake to develop [10]. Therefore we do not believe that naïve astrocytes developed significant effects of TD during this relatively short-term exposure to conditioned media, indicating that soluble factors are more likely responsible for the effects observed.

Previous studies have identified inflammation as an important contributing factor to the cerebral pathophysiology of TD [14,15]. The proinflammatory cytokine TNF-α is a 17-kDa peptide which forms multimers that bind to TNF-α receptors and which are expressed in both neurons and glial cells [16]. TNF-α can be synthesized and released in the brain by astrocytes, microglia, and some neurons and the increased expression and release of TNF-α have been reported in various pathological conditions such as trauma, ischemia, and inflammatory diseases [17–19]. In addition, increased levels of TNF-α in the brain can lead to the activation of NF-κB [20]. Moreover, TNF-α dependent NF-κB activation is also

implicated in the pathogenesis of glutamate neurotoxicity, with increased levels of TNF-α having been shown to inhibit glutamate transport activity in organotypic hippocampal slice cultures [7]. In the present study, we showed that TNF-α dependent activation of NF-κB plays a significant role in the regulation of glutamate transporter function in astrocytes under conditions of TD. NF-κB activation was dependent on TNF-α because cells exposed to TD with an anti-TNF-α antibody blocked the increase in phospho-IκB produced as a result of TD treatment. While TNF-α and NF-κB may contribute to loss of glutamate transporter function in astrocytes with TD, other soluble factors may also produce compromised glutamate transporter integrity in astrocytes exposed to TD conditioned media.

In conclusion, astrocyte-mediated excitotoxicity is an important event in the pathophysiology of TD. Findings from the present study reveal a novel mechanism for the regulation of astrocytic glutamate transporters involving the release of TNF-α and probably other soluble factors from these cells during TD that contribute to decreased glutamate transport capability. Additional studies are necessary to further characterize the nature of the factors released by these cells and their precise role in the loss of glutamate transport function in this disorder.

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