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Transforming growth factor- α mediates estrogen-induced upregulation of glutamate transporter GLT-1 in rat primary astrocytes

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Abstract

Glutamate transporter-1 (GLT-1) plays a central role in preventing excitotoxicity by removing excess glutamate from the synaptic clefts. 17 β -estradiol (E2) and tamoxifen (TX), a selective estrogen receptor modulator (SERM), afford neuroprotection in a range of experimental models. However, the mechanisms that mediate E2 and TX neuroprotection have yet to be elucidated. We tested the hypothesis that E2 and TX enhance GLT-1 function by increasing transforming growth factor (TGF)- α expression and thus, attenuate manganese (Mn)-induced impairment in astrocytic GLT-1 expression and glutamate uptake in rat neonatal primary astrocytes. The results showed that E2 (10 nM) and TX (1 μ M) increased GLT-1 expression and reversed the Mn-induced reduction in GLT-1, both at the mRNA and protein levels. E2/TX also concomitantly reversed the Mn-induced inhibition of astrocytic glutamate uptake. E2/TX activated the GLT-1 promoter and attenuated the Mn-induced repression of the GLT-1 promoter in astrocytes. TGF- α knock-down (siRNA) abolished the E2/TX effect on GLT-1 expression, and inhibition of epidermal growth factor receptor (TGF- α receptor) suppressed the effect of E2/TX on GLT-1 expression and GLT-1 promoter activity. E2/TX also increased TGF- α mRNA and protein levels with a concomitant increase in astrocytic glutamate uptake. All estrogen receptors (ERs: ER- α ER- β and GPR30) were involved in mediating E2 effects on the regulation of TGF- α , GLT-1, and glutamate uptake. These results indicate that E2/TX increase GLT-1 expression in astrocytes via TGF- α signaling, thus offering an important putative target for the development of novel therapeutics for neurological disorders.

Keywords

GLT-1; TGF- α ; glutamate uptake; glutamate transporter; manganese; tamoxifen; 17 β -estradiol; astrocytes

Introduction

17 β -estradiol (E2) exerts neuroprotective effects in the central nervous system (CNS) in addition to its classical roles in regulating neuroendocrine and reproductive function (Singer et al. 1996). Numerous experimental studies demonstrate that E2 exerts neuroprotection in various neurodegenerative disease models (Brann et al. 2011; Shi et al. 1997).

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We have no conflicts of interest.

Epidemiological evidence suggests that E2 has beneficial effects in various neurological conditions, such as Alzheimer's disease (AD) and ischemic stroke (Hurn and Macrae 2000). Despite its neuroprotective properties, E2's clinical utility is limited, given the undesirable side effects from its long-term use, including increased risk of breast and uterine cancers and stroke (Dhandapani et al. 2005). Consequently, selective estrogen receptor modulators (SERMs), such as tamoxifen (TX) and raloxifene, have received extensive attention in attempts to develop agents possessing E2's efficacy, yet eliminating its side effects (Kimelberg et al. 2000; Kokiko et al. 2006).

Previous work has revealed that astrocytes, the major CNS non-neuronal glial cell type, play a critical role in E2-mediated neuroprotection (Azcoitia et al. 2001; Dhandapani and Brann 2007; Spence et al. 2011). Furthermore, growing evidence suggests that E2 protects neurons by modulating synaptic glutamate levels (Pawlak et al. 2005). Elevated glutamate levels are associated with neurotoxic and neurodegenerative processes (Gillissen et al. 2002). Therefore, removal of glutamate from the synapses by specific glutamate transporters, such as glutamate transporter-1 (GLT-1) and glutamate-aspartate transporter (GLAST), is critical for maintaining homeostatic control and optimal synaptic glutamate levels to which neurons are exposed (Gegelashvili and Schousboe 1997). There are five subtypes of glutamate transporters, with GLT-1 and GLAST expressed preferentially in astrocytes (Bridges and Esslinger 2005). E2 increases GLT-1 and GLAST expression and enhances astrocytic glutamate uptake (Lee et al. 2009a; Pawlak et al. 2005).

The mechanisms as to how E2 upregulated GLT-1 and GLAST are unclear. It is known that growth factors play an important role in regulating the expression of glutamate transporters. Along these lines, transforming growth factor (TGF)- β 1, which is neuroprotective (Dhandapani et al. 2005), has been shown to increase GLAST expression (Lee et al. 2009a). In addition, astrocyte-derived TGF- α has been shown to be neurotrophic and neuroprotective (Yuen and Mobley 1996). Intriguingly, astrocytic TGF- α has been shown to be upregulated in response to brain injury (Junier et al. 1991), and induces GLT-1 expression in primary astrocytes (Zelenai et al. 2000), suggesting that increased TGF- α expression increases astrocytic GLT-1 expression and glutamate uptake. Given that E2 increases TGF- α expression in the reproductive system (Mueller et al. 1989; Nelson et al. 1992), we hypothesized that E2 increases GLT-1 expression in astrocytes through TGF- α .

Finally, dysregulation of glutamate transporters has been implicated in the pathology of neurodegenerative disorders. For instance, manganese (Mn) is an essential element, required for normal growth, development and cellular homeostasis; yet chronic exposure to high Mn levels causes neurotoxicity (Cotzias et al. 1968) referred to as manganism, a disease resembling idiopathic Parkinsonism (Barbeau 1984; Sliot et al. 1994). Glutamate-mediated excitotoxicity plays a critical role in Mn neurotoxicity (Hazell 2002). Mn decreases glutamate uptake in astrocytes (Hazell 2002) as well as in Chinese hamster ovary (CHO) cells transfected with GLT-1 and/or GLAST (Mulkus et al. 2005). Given the above observations, we hypothesized that E2 and TX increase and reverse the Mn-induced reduction in astrocytic GLT-1 expression and that this neuroprotective mechanism is mediated by TGF- α . We addressed this hypothesis as well as elaborated the specific receptor mediated mechanisms and signaling pathways involved in E2 and TX actions.

Materials and Methods

Materials

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). TGF- α was from Peprotech (Rocky Hill, NJ). PD98059, LY294002, AG1478 and G-1 were obtained from Tocris Bioscience (Ellisville, MO). L-[3 H]-glutamate was from Amersham Pharmacia

Biotech (Piscataway, NJ). 1,3,5-*tris* (4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) and 2,3-*bis* (4-hydroxyphenyl) propionitrile (DPN) were from Cayman Chemical (Ann Arbor, MI). E2, TX, ICI182,780, DNase, dbc-AMP, protease inhibitors cocktail, poly-D-ornithine and MnCl_2 were from Sigma-Aldrich Corp. (St. Louis, MO). Vectashield mounting medium and DAPI were from Vector Laboratories (Burlingame, CA). Anti-GLT-1, anti-GPR30, anti-TGF- α anti-rabbit and anti-mouse IgG peroxidase conjugates were from Santa-Cruz Biotechnology (Santa Cruz, CA). RNA isolation kit was from Qiagen (Valencia, CA). Luciferase reporter assay kit was obtained from Promega (Madison, WI).

Primary cortical astrocyte cultures

Astrocyte cultures were prepared according to the protocols established previously (Aschner et al. 1992) with minor modifications. Briefly, astrocytes were isolated from the cerebral cortices of 1 day old Sprague-Dawley rats and processed for plating in 6- or 24-well plates (2×10^5 cells/ml) and the media were changed the following day to preserve the adhering astrocytes and to remove neurons and oligodendrocytes. The cultures were maintained at 37°C in a 95% air/5% CO_2 incubator for 3 weeks in Minimal Essential Medium (MEM) with Earle's salts supplemented with 10% heat-inactivated FBS, 100 U/ml of penicillin and 100 $\mu\text{g/ml}$ of streptomycin. The media were changed twice per week. Our experience dictates that the purity of these astrocyte cultures is > 95%-positive for the astrocyte marker, glial fibrillary acidic protein (GFAP) (Lee et al. 2009a). All procedures were carried out in accordance with the Meharry and Vanderbilt IACUC and NIH regulations. All experiments with astrocytes were performed at 3 weeks post isolation, with fully confluent cultures. Three replicates for each cell culture were used for the experiments.

Glutamate uptake assay

Glutamate uptake in astrocytes was measured as previously described (Lee et al. 2009a; Mutkus et al. 2005). After treatment with the designated compounds in opti-MEM reduced serum media, the culture media were replaced with pre-warmed HEPES-buffered solution containing 122 mM NaCl, 3.3 mM KCl, 0.4 mM MgSO_4 , 1.3 mM CaCl_2 , 1.2 mM KH_2PO_4 , 25 mM HEPES, and 10 mM D-(+)-glucose at pH 7.4. After 5 min, pre-warmed uptake buffer containing L-[^3H]-glutamate (0.25 $\mu\text{Ci/ml}$) and unlabeled glutamate was added to achieve a final glutamate concentration of 100 nM. Uptake was terminated after 10 min incubation at 37°C with three washes in ice-cold PBS, immediately followed by cell lysis in 1 ml of 1 N NaOH. An aliquot of 750 μl of sample was neutralized in 75 μl of 10 N HCl, and radioactivity was determined by a liquid scintillation counter. Protein content was determined using a 25 μl aliquot of the sample neutralized in 1 N HCl and quantified by the bicinchoninic acid assay (BCA) protein assay reagent kit (Pierce, Rockford, IL). All levels of radioactivity were corrected for protein levels and calculated as nmol glutamate /mg protein/min.

Western blot analyses

After incubation with the various compounds for the indicated times, astrocytes were washed with cold PBS, then lysed with RIPA lysis buffer containing a protease inhibitor cocktail (10 μl in 1 ml of lysis buffer), followed by protein preparation for electrophoresis. Samples containing 30 μg protein were resolved by SDS-PAGE (10%) and proteins were transferred to nitrocellulose membranes (Whatman, Piscataway, NJ). The membranes were blocked for 1 h at room temperature (RT) in TBS with 0.1% Tween 20 (TBST) containing 5% nonfat milk. The blots were incubated with primary antibodies (GLT-1, 1: 500 or β -actin: 1: 2000) in TBST containing 5% nonfat milk for overnight at 4°C and subsequently washed with TBST, followed by incubation with the secondary antibodies (anti-rabbit or anti-mouse IgG peroxidase conjugates, 1:2000, Santa Cruz Biotechnology) in TBST

containing 5% nonfat milk for 1 h at RT. After washing with TBST, proteins were detected by ECL (Pierce, Rockford, IL).

Immunohistochemistry

After treatments, astrocytes were washed twice with TBS and fixed with 100% methanol for 10 min at RT. After rinsing in TBS, pH 7.4, the cells were incubated for 30 min at RT with TBS containing 5% normal goat serum and 0.1% Triton X-100 (TGT). Cultures were incubated overnight at 4°C in TGT containing rabbit polyclonal anti-GPR30 (1:200), anti-TGF- α and anti-GLT-1 antibodies (TGF- α , 1:250, GLT-1, 1:500). After rinsing in TBS with 0.1% Triton X-100, cells were incubated for 2 h at RT in TGT containing anti-mouse IgG-fluorescein and anti-rabbit IgG rhodamine conjugates (1:200). In separate experiments, primary or secondary antibody was omitted to test for cross-reaction. To visualize cells, cultures were covered with Vector Shield mounting medium and observed under a Nikon confocal microscope (A1R laser scanning confocal, Melville, NY).

Reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA was extracted from astrocytes with the RNeasy mini RNA isolation kit (Qiagen) and 1 μ g total RNA was transcribed to cDNA using poly-dT oligonucleotides and reverse transcriptase. GLT-1 was amplified by PCR using Platinum PCR Supermix (Invitrogen) in a final volume of 30 μ l. The following primers were used: 5'-GGAAGATGGGTGAACAGGC-3' (GLT-1 forward) and 5'-TTCCCACAAATCAAGCAGG-3' (GLT-1 reverse) (110 bp); 5'-TGGAGAACAGCACGTCC-3' (TGF- α forward) and 5'-GCGCTGGGCTTCTCGTG-3' (TGF- α reverse) (363 bp); 5'-TCCCTCAAGATTGTCAGCAA-3' (GAPDH forward) and 5'-AGATCCACAACGGATACATT-3' (GAPDH reverse). After 30 cycles of amplification consisting of denaturing at 94°C for 30 s, annealing at 55–64°C for 30 s and extension at 72°C for 1 min (iCycler, Bio-Rad), samples were electrophoresed on a 1.5% agarose gel and nucleic acids bands were visualized by ethidium bromide staining. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

Real-time RT-PCR

Real-time PCR was performed to amplify GLT-1 using a cycler real-time PCR detection system (Applied Biosystem, Carlsbad, CA). Reactions were carried out in a total volume of 5 μ l containing 2 ng cDNA template of each sample, 0.3 μ M of the appropriate primers and Taqman Mastermix 1 $\times$ (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTP, 50 U/mL iTaq DNA polymerase, 6 mM MgCl₂, Taqman probes, 20 nM fluorescein and stabilizers). The PCR protocol was conducted as follows: 15 s denaturation at 95°C and 1 min annealing at 60°C for 40 cycles. The fluorescence was detected at the end of each elongation step. Each sample was normalized to amplification of GAPDH. Cycle threshold (CT), standard curve preparation and relative quantification of mRNA were calculated by the software (SDS 2.3) provided with the cycler system from Applied Biosystems (Foster City, CA).

siRNA synthesis and transfection

Templates for siRNA synthesis were designed on the basis of the TGF- α sequences such that the total length was 25 bases and the relative %GC of the template was 55%. The sequences of TGF- α used were anti-sense 5'-CAGAUUCCACACUCAGUAUUGUUU-3' and sense 5'-AAACAAUACUGAGUGUGGAAUCUG-3'. These target sequences were previously reported (Yang et al., 2006). Astrocytes were transfected for 3 days, followed by 24 h treatment with E2 (10 nM) or TX (1 μ M) and measurement of protein expression.

Transient transfection and luciferase assay

Astrocytes (1×10^5 cells/well) in 24-well plates were cultured in 500 μ l of regular growth medium for 2-3 days before transfection. The plasmid vector, pGL3 containing EAAT2Pro-954, was kindly provided by Dr. Fisher, Virginia Commonwealth University. Medium was replaced with MEM containing 5% FBS and transfected overnight with 0.5 μ g of reporter plasmid with lipofectamine 2000 (Invitrogen, Carlsbad, CA). After overnight transfection, opti-MEM containing the designated treatment was replaced and cells were incubated for 24 h. Luciferase activity was measured with the Bright-Glo luciferase kit (Promega, Madison, WI). Luciferase activity was normalized to protein content. In all experiments, the normalization procedure was verified by co-transfecting cells with the pGL4.75 plasmid carrying the renilla luciferase reporter gene and the dual luciferase assay system (Promega, Madison, WI).

Statistical analysis

The mean and standard error of the mean (S.E.M.) were determined for each set of data and one-way analysis of variance (ANOVA) followed by Tukey post-hoc test was used for statistical analysis to compare control and treated groups with statistical significance set at $p < 0.05$. Each experiment was carried out in three or more independently isolated primary astrocyte preparations, with n as the statistical unit representing the number of cultures used (not wells). The number of samples for each experiment was at least three for western blot and real time-PCR analyses and four for glutamate uptake assays. Data analyses were carried out with GraphPad software (Graph Pad, San Diego, CA).

Results

E2 and TX reversed the Mn-induced reduction in GLT-1 protein expression

We first examined the ability of E2 and TX to reverse Mn-induced suppression of GLT-1. Treatment of neonatal rat astrocyte cultures with Mn (6 h) at 125, 250 or 500 μ M significantly decreased astrocytic GLT-1 protein expression in a concentration-dependent manner by 25 ± 3 , 41 ± 2 and $49 \pm 3\%$, respectively (Fig. 1A). The physiological Mn range (which produces no symptoms) is ~ 75 -100 μ M, and clinical signs increase in frequency and severity above this level, suggesting this concentration is near the threshold of toxicity (Suzuki et al. 1975). Therefore, a 250 μ M Mn concentration is relevant and was chosen for the present studies.

To determine the concentrations of E2 and TX to use for our mechanistic studies, we measured the effects of a range of concentrations of each on GLT-1 expression. E2 (5, 10, 20 nM) and TX (1, 2 μ M) significantly increased astrocytic GLT-1 protein levels by 36 ± 4 , 49 ± 2 and $44 \pm 3\%$ for E2, and 43 ± 2 and $29 \pm 3\%$ for TX, respectively (Fig. 1B). E2 at 10 nM and TX 1 at μ M were the most effective in increasing astrocytic GLT-1 protein expression after 24 h incubation (Fig. 1B). Moreover, 18 h pretreatment with E2 (5, 10, 20 nM) or TX (1 μ M) reversed the 6 h Mn (250 μ M)-induced reduction in astrocytic GLT-1 expression (Fig. 1C). Immunocytochemistry corroborated the Mn-induced reduction in astrocytic GLT-1 expression and the ability of both E2 and TX to reverse this effect (Fig. 1D). Furthermore, the reversal by E2 and TX of the Mn-induced inhibition of GLT-1 expression was paralleled by the ability of E2 and TX to reverse the Mn-induced inhibition of astrocytic glutamate uptake (Fig. 1E). Given these observations, the time-course for the remainder of the experiments consisted of pretreatment with E2 or TX for 18 h followed by 6 h Mn exposure (250 μ M) in the presence of E2 or TX.

E2 and TX reversed the Mn-induced reduction in GLT-1 mRNA

To better characterize putative mechanisms by which Mn, as well as E2 or TX, modulate astrocytic GLT-1 expression, we analyzed GLT-1 transcriptional levels by RT-PCR and real time-PCR. Astrocytic GLT-1 mRNA levels were significantly increased by both E2 (10 nM; $p<0.001$) and TX (1 μ M; $p<0.05$), whereas its expression was significantly decreased ($p<0.001$) by 6 h Mn treatment (Fig. 2A and 2B). Pretreatment with E2 or TX prior to Mn exposure reversed the Mn-induced reduction in GLT-1 mRNA expression ($p<0.01$; Fig. 2B).

E2 and TX reversed Mn-induced repression of the GLT-1 (EAAT2) promoter

Next we tested whether E2 or TX can activate the GLT-1 promoter. Astrocytes were transiently transfected with an EAAT2 promoter-driven luciferase reporter vector (EAAT2Pro-954). The full sequence of EAAT2 promoter is 2417 bases; the p-954 deletion mutant (EAAT2Pro-954) exerts the equivalent promoter activity to the full EAAT2 promoter sequence (Su et al. 2003). EAAT2, the human astrocytic glutamate transporter, shares 76% sequence homology with the rat GLT-1 by basic local alignment search tool (BLAST) and shows analogous regulation to rats' GLT-1 (Allritz et al. 2010).

EAAT2 (GLT-1) promoter activity was increased approximately 2-fold by either E2 or TX (Fig. 3A). G-1, a selective agonist for the G-protein coupled receptor 30 (GPR30) of estrogen, and 4-hydroxytamoxifen (4-OHTX), an active metabolite of TX, were also tested to gain a better understanding on EAAT2 promoter activity regulation by estrogen. As shown in Fig. 3, E2, TX, 4-OHTX and G-1 (20 h treatment) significantly ($p<0.01$) increased the astrocytic EAAT2 promoter activity. Pretreatment with E2 or TX prior to Mn exposure reversed the Mn-induced repression in GLT-1 promoter activity (Fig. 3B). To further characterize the mechanism of E2 and TX-induced GLT-1 activation, inhibitors of the E2 signaling pathways were tested.

While ICI is an antagonist of the genomic ER- α/β , earlier studies reported that it exerted agonistic effects on GPR30 in several cell types (Filardo et al. 2002). Notably, ICI increased ($p<0.01$) EAAT2 promoter activity (Fig. 3C), indicating ICI exerts agonistic effect on GLT-1 (EAAT2) regulation, possibly via GPR30.

Moreover, blocking the EGFR pathway with AG1478 or the PKA pathway with H89 completely abolished the effects of these estrogenic compounds on EAAT2 promoter activity (Fig. 3C). E2/TX-enhanced EAAT2 promoter activity was also blocked by PI3K inhibitor LY294002 (Fig. 3D).

Astrocytic TGF- α expression is increased by E2 and TX and decreased by Mn

Since the GLT-1 (EAAT2) promoter region does not contain a *cis*-element for an estrogen response element (ERE) motif, the E2/TX-activated estrogen receptor (ER) complex is unlikely to directly activate this promoter. Therefore, we hypothesized that TGF- α mediates the E2-induced activation of the GLT-1 promoter because E2 is known to increase TGF- α expression (Ma et al. 1994), which increases GLT-1 expression (Zelenaia et al. 2000).

As shown in Fig. 4, E2 and TX significantly increased both TGF- α protein ($p<0.001$ for E2 10 nM, $p<0.01$ for TX 1 μ M) (Fig. 4A) and mRNA levels ($p<0.05$ for E2 10 nM and TX 1 μ M) (Fig. 4D), whereas Mn decreased TGF- α protein levels ($p<0.001$) (Fig. 4B). Pretreatment (18 h) with E2 or TX reversed the Mn-induced inhibition of astrocytic TGF- α expression (Fig. 4B). Immunostaining corroborated these findings (Fig. 4D). These results indicate a putative role for TGF- α in mediating the effect of E2 and TX on GLT-1 expression. This is also supported by the result that inhibition of EGFR with AG1478 abolished E2 and TX-induced activation of the GLT-1 (EAAT2) promoter (Fig. 3C).

TGF- α increased GLT-1 expression and glutamate uptake in astrocytes

If TGF- α mediates the effect of E2 and TX, astrocytes must express its receptor, EGFR, and TGF- α itself should increase GLT-1 expression. Expression of EGFR was confirmed by RT-PCR and immunocytochemical staining (Fig. 5A). Treatment (24 h) with TGF- α (50 ng/ml) significantly increased GLT-1 expression ($p < 0.01$) (Fig. 5B) and glutamate uptake ($p < 0.001$) (Fig. 5C), and reversed the Mn-induced inhibition of glutamate uptake (Fig. 5C).

Astrocytic TGF- α silencing with siRNA abolished the effect of E2 and TX on GLT-1 expression

To determine whether TGF- α mediates the effect of E2 and TX on GLT-1 expression, TGF- α was knocked-down by siRNA transfection. Astrocytes were transfected with TGF- α siRNA for 3 days prior to 24 h treatment with E2 or TX, followed by western blot analysis. Suppression of TGF- α expression was confirmed by RT-PCR 3 days post transfection. As shown in Fig. 6A, TGF- α mRNA levels were significantly suppressed ($p < 0.05$). The effect of E2 or TX on GLT-1 expression was completely abrogated when TGF- α was knocked-down (Fig. 6B) as shown by western blot analysis (Fig. 6C).

EGFR, MAPK, PI3K and EGFR are involved in E2 and TX-induced GLT-1 expression

E2 and TX stimulate the phosphorylation of astrocytic MAPK and PI3K (Lee et al. 2009b). Therefore, we tested whether these pathways play a role in E2- and TX-induced enhancement of GLT-1 expression. As shown in Fig. 7, PD98059, a specific inhibitor of the MAPK pathway, completely blocked the E2- and TX-induced increase in GLT-1 expression (Fig. 7A and 7B). Inhibition of the PI3K/Akt pathway with LY294002 also suppressed the E2-induced GLT-1 expression, but partially suppressed the TX effect on GLT-1 (Fig. 7A and 7B). Since TGF- α mediates E2- and TX-induced GLT-1 expression (Fig. 6), EGFR pathway is considered crucial for their effects on GLT-1 expression. The role of EGFR pathway was determined by testing AG1478, a specific inhibitor of EGFR, which abolished the E2-induced increase in GLT-1 expression (Fig. 7A and 7B).

Role of subtypes of the ERs, ER- α , ER- β and GPR30 in GLT-1 expression and glutamate uptake

Next, we determined which subtypes of the ER (ER- α , ER- β or GPR30) mediate the astrocytic GLT-1 induction in response to E2 and TX. That astrocytes express ER- α and ER- β is already known; to determine whether GPR30 might contribute to E2 and TX effects, we first confirmed its expression (Fig. 8A). ER agonists (PPT for ER- α , DPN for ER- β and G-1 for GPR30) increased GLT-1 expression (Fig. 8A) and glutamate uptake (Fig. 8B). PPT and DPN were tested at concentrations up to 800 pM (Zhao and Brinton 2007; Zhao et al. 2004) and significantly increased glutamate uptake ($p < 0.001$) (Fig. 8C and 8D). The contribution of each ER subtype to astrocytic glutamate uptake was also tested to determine whether they act synergistically or additively. The results showed that the GPR30 pathway is likely the main regulator of GLT-1 expression in response to E2 (Fig. 9). G-1 (4 μ M) exerted the highest efficacy in increasing glutamate uptake; PPT and DPN also significantly increased glutamate uptake, but to a lesser extent than G-1 ($p < 0.001$) (Fig. 9). Interestingly, glutamate uptake was not substantially enhanced by combined G-1 (4 μ M) and PPT 400 pM treatments (Fig. 9A and 9B), suggesting they act through the same signal transduction pathway. Moreover, co-treatment with PPT and DPN did not enhance uptake to the same degree as G-1 (4 μ M) (Fig. 9C).

Effect of various SERMs on GLT-1 and glutamate uptake

Next we determined whether co-treatments of multiple estrogenic compounds exert additive, synergistic or antagonistic effects on GLT-1 protein expression and glutamate

uptake. G-1 is known to be highly selective for GPR30, exerting no binding activity to other ERs (ER- α and ER- β) and no activation of ERE (estrogen response element) reporter at its concentration of up to 10 μ M (Dennis et al. 2011). When individually applied, each SERM increased astrocytic GLT-1 protein levels (Fig. 10A) and glutamate uptake (Fig. 10B). G-1 (4 μ M) showed the highest efficacy. Interestingly, when G-1 was co-treated with E2 (10 nM), TX (1 μ M) or ICI (1 μ M), GLT-1 protein and glutamate uptake failed to increase in an additive manner (Fig. 10A and 10B), indicating that all these compounds might use a same pathway in downstream of their receptor activations for GLT-1 regulation. Co-treatment with E2 and TX resulted in additive effects on GLT-1 protein expression and glutamate uptake activity (Fig. 10B).

Discussion

The present study demonstrates that E2-induced increases in astrocytic GLT-1 protein and mRNA expression, as well as glutamate uptake, are mediated by TGF- α . Although TX exerts antagonistic effects in the breast tissue, inhibiting E2 action in E2-dependent breast cancer, TX exerts an agonistic effect on GLT-1 expression in astrocytes. We believe that all three ERs may mediate E2 action on GLT-1 as selective agonists for ER α , ER β and GPR30 induced upregulation on GLT-1 and glutamate uptake.

The present study also establishes the efficacy of E2 and TX in attenuating Mn-induced inhibition of GLT-1 mRNA, protein expression and glutamate uptake (Fig. 1 and 2). Since GLT-1 is a critical astrocytic glutamate transporter that maintains optimal synaptic glutamate concentrations, understanding the mechanisms by which E2 and TX reverse the Mn-induced impairment in GLT-1 expression should provide invaluable information in the search of novel targets for developing neuroprotective therapeutics.

Astrocytes produce a variety of neuroactive substances and neurotrophic factors (Barres 1991; Martin 1992). Although both epidermal growth factor (EGF) and TGF- α interact with EGFR to exert their cellular actions, TGF- α expression is more prevalent than EGF, indicating that the former is the predominant EGFR ligand in the CNS (Lazar and Blum 1992). TGF- α is particularly abundant in astrocytes (Ma et al. 1994) and its expression has previously been shown to increase in response to E2 (Ma et al. 1994). Notably, E2 levels and TGF- α expression are increased in response to injury (Garcia-Segura et al. 1999; Junier et al. 1991). E2 is also known to induce TGF- α expression by activating the TGF- α promoter via functional palindromic estrogen response element (ERE)-like sequences (Blasband et al. 1990; El-Ashry et al. 1996). TGF- β 1 mediates the enhancing effect of E2 or TX on upregulation of GLAST in astrocytes as noted in our previous study (Lee et al., 2009a). Nevertheless, the precise mechanism by which E2/TX regulates glutamate transporters via TGF- α and TGF- β 1 has yet to be delineated. We found that both TGF- α and TGF- β 1 activate the MAPK and PI3K pathways, yet these two factors activate distinct receptors (TGF- α activates EGFR, while TGF- β 1 activates TGF beta receptor I and II). E2/TX is likely involved in complicated network pathways to exert glutamate transporter enhancement via these transforming growth factors.

Given that inhibition of EGFR abolished the ability of E2/TX to stimulate GLT-1 protein expression in cortical astrocytes, which inherently express E2 receptors (ERs), it is likely that E2 and TX enhance GLT-1 expression via the TGF- α /EGFR pathway. The significance of this action is supported by the previous reports that astrocytic TGF- α production and release induces neuronal survival (Hsieh and Conti 2005).

The mechanism responsible for E2-mediated TGF- α induction and subsequent GLT-1 protein expression has yet to be defined. The promoter region of GLT-1 does not contain

estrogen responsive elements (EREs) (Su et al. 2003), indicating that the E2-induced enhancement of astrocytic GLT-1 expression (Fig. 1B) reflects indirect activation of the GLT-1 promoter. Therefore, we propose that TGF- α -induced by E2 leads to increased GLT-1 expression, consistent with the observation that suppression of TGF- α with siRNA abolishes the ability of E2/TX to enhance GLT-1 protein expression.

Our study further demonstrated that E2-induced astrocytic GLT-1 expression is mediated by MAPK and PI3K pathways (Fig. 7), while TX partially suppressed PI3K, suggesting that TX likely acts through mechanisms distinct from E2. The role of the nuclear ERs, ER- α and ER- β , in mediating GLT-1 expression is supported by the observations that the ER- α/β selective agonists, PPT and DPN, enhanced GLT-1 expression and glutamate uptake (Fig. 8). This nuclear ER- α/β -induced TGF- α may regulate EGFR signaling by an autocrine mode via the plasma membrane EGFR (Levin 2003). The non-genomic ER, G protein-coupled GPR30, is known to transactivate EGFR and ERK signaling (Vivacqua et al. 2009). Since the GPR30 pathway may act via mechanisms distinct of the genomic action of E2-induced effects and does not regulate reproductive system, it has garnered special attention as a potential target for novel pharmacotherapeutic approaches in both cancer and neurological disorders (Lebesgue et al. 2010; Lin et al. 2009). The present study shows that activation of GPR30 by administration of G-1, a selective agonist of GPR30, increased GLT-1 expression, suggesting the role of GPR30 in regulation of glutamate transporters.

GLT-1 (EAAT2 in human) gene expression is regulated by multiple transcription factors (Su et al. 2003). TGF- α , analogous to EGF, is known to activate the GLT-1 promoter via the NF- κ B pathway (Sitcheran et al. 2005). The GLT-1 promoter activation by both E2 and TX was suppressed by EGFR inhibition (Fig. 3), indicating that TGF- α likely mediates E2 and TX's enhancement of glutamate transporter expression via GLT-1 promoter activation secondary to stimulation of the EGFR-NF- κ B pathway. The GLT-1 promoter also contains a CREB binding motif, and TGF- α activates the CREB pathway (Schluter et al. 2002). This conclusion is supported by observations that the inhibition of PKA abolished E2's and TX's effects on EAAT2 (GLT-1) promoter activity (Fig. 3) and mutation in the Cre site of the GLT-1 promoter significantly decreased GLT-1 promoter activity (unpublished data). Taken together, these results suggest that E2 and TX-induced TGF- α activates not only NF- κ B, but also CREB binding sites in the GLT-1 promoter.

The present study suggests that TGF- α is likely involved in mediating Mn's effect on GLT-1, consistent with the significant suppression of astrocytic TGF- α expression ($p < 0.01$) by Mn (Fig. 4B). Moreover, E2 and TX attenuated the Mn-reduced TGF- α expression, indicating that TGF- α might mediate the E2 and TX reversal of Mn-induced GLT-1 impairment. E2 and TX reversal of the Mn effect on GLT-1 expression may be mediated by blocking Mn-induced GLT-1 impairment or by enhancing GLT-1 expression. The latter mechanism is supported by observations that E2 and TX are able to increase GLT-1 expression via TGF- α upregulation (Fig. 6). Nonetheless, since Mn is known to induce oxidative stress, other mechanisms, such as anti-oxidant responses, may also play a role in the ability of E2 and TX to attenuate the Mn-induced impairment in GLT-1 expression (Hazell et al. 2010; Lee et al. 2009b).

Our finding suggests that SERMs, such as TX, or GPR30 agonists, such as G-1, may proficiently regulate GLT-1 expression and afford neuroprotection. In particular, G-1 via GPR30 seems intriguing to be explored for its detailed mechanism in GLT-1 expression since it induced the highest efficacy in GLT-1 expression and glutamate uptake among all ERs (Fig. 9 and 10). G-1 exerts no significant binding to either ER α or ER β at concentrations as high as 10 μ M and yet, might bind ER α as a low-affinity antagonist (Dennis et al. 2009). G-1 thus, has typically been used to determine the contribution of

GPR30 activation to a cellular or physiological response in the absence of estrogen. It has been reported that TX and G-1 exert neuroprotective effects in several experimental models (Lebesgue et al. 2010; Sharma and Mehra 2008; Wakade et al. 2008). For example, ICI, an antagonist of the genomic ER- α/β , exerts agonistic effects on GPR30 in several cell types (Filardo et al. 2002; Thomas et al. 2005). It has also been reported that ICI exerts neuroprotective effects in neuronal cells (Zhao et al. 2006), consistent with our finding that ICI exerts agonistic effect on GLT-1 expression, possibly via GPR30 activation. Because the ability of TX and G-1 to prevent injury is so well established, it seems likely that this study will contribute to a better understanding of mechanisms by which E2/SERMs, including TX and G-1, enhance GLT-1 expression and might lead to the development of novel therapeutics for neurodegenerative diseases.

In summary, results from the present study establish that (1) E2 and TX reverse the Mn-induced reduction in astrocytic GLT-1 expression and glutamate uptake; (2) TGF- α mediates the effect of E2 and TX on GLT-1; (3) the genomic ERs, ER- α and ER- β , as well as the non-genomic ER, GPR30, play a role in the E2/TX effects on GLT-1; and (4) the PKA pathway is involved in mediating E2's and TX's effects on GLT-1 expression. These results are summarized in Fig. 11 as a proposed mechanism of E2/TX action on GLT-1 expression.

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Fig. 1. E2 and TX reverse the Mn-induced impairment in astrocytic glutamate transporter GLT-1. Astrocytes were (A) exposed to Mn (0-500 μM) for 6 h, (B) treated with E2 or TX for 24 h, or (C) preincubated with E2 or TX for 18 h prior to exposure to Mn (250 μM) for 6 h. After

treatment, cell lysates were subjected to western blot analysis to detect GLT-1 protein expression. (D), For [^3H]glutamate uptake, astrocytes were treated as described in (C). (E), For Immunostaining for GLT-1, astrocytes were treated for 6 h with Mn, E2 or TX for 24 h, and E2 or TX for 18 h prior to 6-h Mn treatment. Data are expressed as mean $\pm$ SEM (N=4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n=4).

Fig. 2.

E2 and TX reverse the Mn-induced reduction in astrocytic GLT-1 mRNA levels. (A) Astrocytes were incubated with E2 or TX for 24 h and RNA was isolated for conventional RT-PCR and real time-PCR. (B) Astrocytes were preincubated for 18 h with E2 or TX prior to 6 h treatment with Mn (250 μM) followed by RT-PCR and real time-PCR assays. Data are expressed as mean ± SEM (N=4). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (n=4).

Fig. 3.

E2 and TX reverse Mn-induced repression of the GLT-1 (EAAT2) promoter. Astrocytes were transfected with the EAAT2pPro-954 together with a renilla luciferase vector as an internal control. dbcAMP was used as a positive control. After transfection for overnight, astrocytes were treated with various compounds for 20 h followed by luciferase assay. (A) Astrocytes were treated with E2 (10 nM), TX (1 μ M), 4-OHTX (1 μ M), G-1(1 μ M) and ICI (1 μ M) activated the EAAT2 (GLT-1) promoter. (B) Cells were pretreated for 18 h with E2 (10 nM) or TX (1 μ M) prior to Mn exposure for 6 h in the presence of E2 or TX. (C) Cells were preincubated for 30 min with inhibitors of EGFR (AG1478, 10 μ M) and PKA (H89, 10 μ M) prior to 24 h treatment with E2, TX or ICI. (D) Cells were preincubated for 30 min with a PI3K inhibitor (LY294002, 20 μ M) prior to 24 h treatment with E2 or TX. Results are presented as the mean $\pm$ SEM of the percent induction compared to the control. * p <0.05, ** p <0.01, *** p <0.001 (n =4).

Fig. 4.

E2 and TX reverse the Mn-induced reduction in TGF- α protein expression. (A) Astrocytes were treated for 24 h with E2 or TX and TGF- α protein levels were examined by western blot analysis. (B) Astrocytes were preincubated for 18 h with E2 or TX prior to 6 h Mn exposure, followed by TGF- α protein analysis. (C) Immunocytochemical staining using same treatment paradigm as in previous experiments. (D) TGF- α expression (real-time and RT-PCR) following 24 h of E2 or TX treatment, or 18 h pretreatment with E2 or TX followed by 6 h Mn treatment in the presence of E2 or TX. Data are expressed as mean $\pm$ SEM (n=3). *p<0.05, **p<0.01, ***p<0.001.

Fig. 5. TGF-α reverses the Mn-induced reduction in glutamate transporter function. (A) Astrocytes express TGF-α and EGFR. (B) GLT-1 expression following incubation of astrocytes with TGF-α for 24 h. (C) Glutamate uptake following either 24 h TGF-α treatment or 6 h Mn treatment followed by 18 h TGF-α preincubation. Data are expressed as mean ± SEM (N=4). *p<0.05, **p<0.01, ***p<0.001.

Fig. 6.

TGF- α siRNA abolishes E2- and TX-induced GLT-1 expression in rat primary astrocytes. Astrocytes were transfected for 3 days with negative control scrambled RNA (sc RNA) or TGF- α siRNA. (A) RT-PCR for TGF- α ; GAPDH used as a loading control. After 3 days of transfection with TGF- α siRNA, astrocytes were treated for 24 h with E2 or TX using scrambled siRNA as a negative control. (B), Western blotting for GLT-1 and quantitative analysis of the band densities. Data are expressed as mean $\pm$ SEM (N=4). ** p <0.01, *** p <0.001.

Fig. 7.

MAPK, PI3K and EGFR pathways are involved in E2- or TX-induced GLT-1 expression in astrocytes. Astrocytes were pre-treated for 30 min with 50 μM PD98059 (MAPK inhibitor), 20 μM LY294002 (PI3K inhibitor), or 10 μM typhostin AG1478 (EGFR kinase inhibitor) prior to 24 h treatment with E2 or TX in the presence of the inhibitors, followed by western blotting for GLT-1. Data are expressed as mean ± SEM (N=3). **p<0.01, ***p<0.001.

Fig. 8.

All ER subtypes contribute to E2-induced GLT-1 expression and uptake. (A) GPR30 mRNA and protein are inherently expressed in astrocytes. (B) G-1 (a selective agonist for GPR30), PPT (a selective agonist for ER- α), or DPN (a selective agonist for ER- β) increased astrocytic GLT-1 expression upon 24 h incubation. (C-D) Glutamate uptake in astrocytes following treatment with (C) PPT or (D) DPN. Data are expressed as mean $\pm$ SEM (N=4). * p <0.05, ** p <0.01, *** p <0.001.

Fig. 9.

GPR30 contributes more to astrocytic glutamate uptake than other ER subtypes. Astrocytes were treated with (the highest effective concentrations of each compound for glutamate uptake) (A) G-1 (4 μ M) and PPT (400 pM), (B) G-1 (4 μ M) and DPN (400 pM), or (C) PPT and DPN for 24 h, and glutamate uptake was analyzed. Data are expressed as mean $\pm$ SEM (N=4). * p <0.05, ** p <0.01, *** p <0.001.

Fig. 10.

Various ER ligands have additive effects on GLT-1 expression and glutamate uptake; no treatment could increase the enhancement caused by G-1 4 μ M. Astrocytes were treated for 24 h with G-1 (4 μ M) alone or with various ER ligands (E2 10 nM, TX 1 μ M, or ICI 1 μ M) in astrocytes, followed by western blot and glutamate uptake analyses. Data are expressed as mean $\pm$ SEM (N=4). * p <0.05, ** p <0.01, *** p <0.001.

Fig. 11.

Proposed mechanism for the effect of E2 and TX on GLT-1 expression. E2 and TX increase GLT-1 expression via the nuclear ER (ER-α and ER-β) as well as GPR30. GPR30 resides on either the plasma membrane and/or endoplasmic reticulum. E2-or TX-induced TGF-α is released into the extracellular space and activates EGFR by autocrine/paracrine modes. TGF-α mediates E2 and TX action on GLT-1 expression by multiple signaling pathways, including EGFR, PI3K, MAPK and PKA.