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Mechanism of Raloxifene-induced Upregulation of Glutamate Transporters in Rat Primary Astrocytes

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Abstract

Raloxifene (RX), a selective estrogen receptor modulator (SERM), exerts neuroprotection in multiple clinical and experimental settings. Astrocytic glutamate transporters GLT-1 (EAAT2) and GLAST (EAAT1) are the main glutamate transporters in the central nervous system, taking up most of excess glutamate from the synaptic cleft to prevent excitotoxic neuronal death. Since drugs targeting astrocytic glutamate transporters to enhance their expression and function represent potential therapeutics for neurodegenerative disorders associated with excitotoxicity, we tested if RX modulates the expression and function of GLT-1 and GLAST in rat primary astrocytes. The results showed that RX significantly increased glutamate uptake and expression of GLT-1 mRNA and protein levels. RX enhanced GLT-1 expression by the activation of multiple signaling pathways including ERK, EGFR and CREB mediated by estrogen receptors (ERs) ER- α , ER- β and GPR30. At the transcriptional level, NF- κ B played a critical role in RX-induced GLT-1 expression as RX increased NF- κ B reporter activity and induced binding of NF- κ B p65 and p50 to the GLT-1 promoter. RX attenuated the reduction of GLT-1 expression and glutamate uptake induced by manganese (Mn) whose chronic high levels of exposure cause manganism. RX also upregulated GLAST by increasing its promoter activity and protein levels via the NF- κ B pathway and ERs. Our findings provide new insight into the mechanism of RX-induced enhancement of GLT-1 and GLAST expression, as well as the attenuation of Mn-reduced expression of these transporters. These findings will be highly valuable for developing therapeutics of neurodegenerative diseases associated with impaired astrocytic glutamate transporters.

Keywords

raloxifene; GLT-1; GLAST; manganese; estrogen receptors; NF- κ B

Introduction

Although 17 β -estradiol (E2), a female sex hormone, is mainly involved in reproductive development, numerous studies in recent years have reported that E2 exerts neuroprotective effects in experimental models as well as in clinical studies [reviewed in (Brann et al.,

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2007)]. However, its clinical application for neuroprotective purposes has been hampered due to its adverse effects such as increasing the risk of breast, lung and uterine cancers, thrombosis and stroke (Canonica et al., 2008; Persson et al., 1999; Scarabin-Carre et al., 2012; Slatore et al., 2010). This limitation led to the search for specific estrogenic compounds which can selectively target the brain tissue without unwanted peripheral effects. Developing selective estrogen receptors modulators (SERMs) as therapeutics for specific diseases is of great interest due to their selective estrogen agonistic and antagonistic activities depending on the tissue types (DonCarlos et al., 2009; Lewis and Jordan, 2005; Littleton-Kearney et al., 2002). Ideal neuroSERMs would exert agonistic effects specifically on the brain tissue without exerting undesirable side effects on other tissues (Brinton, 2002; Zhao et al., 2005). Thus understanding the mechanisms underlying SERM-induced neuroprotection is imperative.

It appears that both genomic pathways via classical estrogen receptors (ERs), ER- α and ER- β , acting as transcription factors, and non-genomic signaling pathways mediate the neuroprotective effects of E2 and SERMs. The non-genomic pathway activates multiple intracellular signaling proteins such as MAPK, PI3K/Akt, Src and CREB, which are involved in SERM-induced neuroprotective effects (Dhandapani and Brann, 2002; Lebesgue et al., 2009; Lee et al., 2009b). In addition, the anti-oxidative action of SERMs also contributes to their neuroprotection in an ER-independent manner (Behl et al., 1997; Zhang et al., 2007).

Dysregulation of astrocytic glutamate transporters, in particular glutamate transporter-1 (GLT-1) which takes up most of excess glutamate from the synaptic cleft to maintain optimal glutamate levels, is closely associated with excitotoxic neuronal injury (Ullensvang et al., 1997) and neurodegenerative diseases (Danbolt, 2001). Therefore, compounds that induce enhancement of glutamate transporter expression and function will offer therapeutic potential for these disorders (Kim et al., 2011). E2 as well as SERMs can exert neuroprotection via direct regulation of neuronal functions or/and indirect modulation of astrocytic functions (Dhandapani and Brann 2002; Kajta and Beyer, 2003). In support of the role of astrocytic glutamate transporters in E2/SERMs neuroprotection, in rodent primary astrocytes, E2 and tamoxifen (TX) increase GLAST and GLT-1 mRNA and protein levels in astrocytes (Karki et al., 2013; Lee et al., 2009a; Pawlak et al., 2005) and attenuate the manganese (Mn)-induced impairment of these transporters by non-genomic ER signaling pathways such as ERK/MAPK, PI3K/Akt and EGFR (Lee et al., 2012b; Lee et al., 2009a).

Raloxifene (Evista, RX) is a non-steroidal benzothiophene SERM approved by the FDA to treat and prevent osteoporosis in menopausal women and invasive breast cancer due to its agonistic effect on bone and its antagonistic effect on breast tissue (Clemett and Spencer, 2000; Moen and Keating, 2008). When used for breast cancer therapy, RX is considered a safer drug than TX due to its antagonistic effect on uterine tissues in contrast to TX which causes uterine cancers due to its agonistic effect in uterine tissues (D'Amelio and Isaia, 2013; Vogel et al., 2006). Interestingly, like TX, several studies have reported that RX also exerts beneficial effects in the brain. RX significantly improved verbal memory in healthy postmenopausal women of 70 years or older (Jacobsen et al., 2010) and reduced the risk of mild cognitive impairments as well as dementia in postmenopausal osteoporotic women

(Yaffe et al., 2005). Several *in vitro* and *in vivo* experimental studies have also demonstrated that RX induces neuroprotection in various animal models of neurodegenerative disorders (Arevalo et al., 2011). Nonetheless, the molecular and cellular mechanisms involved in RX-induced neuroprotection remain to be elucidated, although recent studies suggest that RX-induced neuroprotection involves anti-inflammatory and anti-apoptotic actions and a reduction of excitotoxicity (Cerciat et al., 2010; Ciriza et al., 2004; Du et al., 2004). Further studies are required to better understand the molecular mechanisms involved to identify the precise molecular target(s) of RX neuroprotective actions.

In the present study, we investigated if RX enhances astrocytic glutamate transporters and if so, what molecular mechanisms are involved. Our findings demonstrate that RX enhances both GLT-1 and GLAST protein levels as well as glutamate uptake via multiple signaling pathways including PKA, MAPK, EGFR and NF- κ B in astrocytes.

Materials and Methods

Materials

RX, MnCl₂ and 3,4,5-dimethyl thiazol-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO). The cell culture media such as MEM, DMEM and Opti-MEM, and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA). [³H]Glutamic acid was from PerkinElmer. UCPH 101, AG1478, pyrrolidine dithiocarbamate (PDTC), H89, PP2, LY294002 (LY), PD98059 (PD), dihydrokainate (DHK) and G15 were from Tocris Bioscience (Ellisville, MO). GLAST (ab 416) and GLT-1 (ab 58571) antibodies were from Abcam (Cambridge, MA). ERK (sc-135900), phospho-ERK (sc-7383), Akt (sc-55523), phospho-Akt (sc-7985), CREB (sc-186), phospho-CREB (sc-7978), EGFR (sc-120), phospho-EGFR (sc-12357), NF- κ B (p65, sc-372 and p50, sc-114), EAAC1 (sc-25658) and β -actin (sc-1616) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). A luciferase reporter assay kit was obtained from Promega (Madison, WI).

Primary Cultures of Astrocytes

Astrocytes were isolated from cortices of 1-day-old Sprague–Dawley rats as previously described (Aschner et al., 1992). Briefly, after carefully removing the meninges, cerebral cortices were digested with dispase and astrocytes were then recovered by the repeated removal of dissociated cells and plated at a density of 1×10^5 cells/mL. The cultures were maintained at 37°C in a 95% air, 5% CO₂ incubator for 3 weeks in minimum essential medium (MEM) supplemented with 10% horse serum, 100 U/mL of penicillin and 100 μ g/mL of streptomycin. These cultures showed > 95%-positive staining for the astrocyte-specific marker, glial fibrillary acidic protein (GFAP). All experiments were performed 3 weeks post-isolation.

Primary Cultures of Neurons

The primary cultures of neurons were prepared from cortices of rat embryos on day 18 of gestation as previously described (Behar et al., 1999). The cells were seeded in poly-L-lysine coated 6-well plates with 1×10^6 cells/well in Neurobasal media (Gibco) supplemented

with 5% fetal bovine serum, 5% horse serum, 1% penicillin/streptomycin and 1 mM glutamine. After two days, cytosine arabinoside (2.5 μ M) was added to the growing cells to inhibit the proliferation of non-neuronal cells. All experiments were completed within 10–12 days of the neuronal cultures.

Reagent Preparation and Cell Treatment

RX, AG, H89, PDTC, PD, G15, ICI, DPN and PPT were dissolved in dimethyl sulfoxide (DMSO, <0.5% in a final solution) and diluted with Opti-MEM. $MnCl_2$ was dissolved in Hanks's balanced salt solution (HBSS). Growth media for primary astrocytes were replaced with experimental media (Opti-MEM) prior to adding designated compounds for the experiments. One μ M concentration of RX, G15 and ICI, and 200 pM concentration of PPT and DPN were used. In some experiments, cells were pre-treated with PDTC (50 μ M), PD (50 μ M), LY (20 μ M) or AG (10 μ M) for 30 min prior to adding RX, PPT or DPN for 24 h incubation. For Mn and RX treatment, cells were treated with RX (1 μ M) for 18 h prior to Mn (250 μ M) exposure for 6 h.

SDS-PAGE and Western Blot

Astrocytes treated with the designated compounds for the indicated time periods were washed twice with cold phosphate buffered solution (PBS). The cells were then lysed with radio immunoprecipitation assay buffer (RIPA) containing protease inhibitor cocktail (Sigma-Aldrich). The protein concentration in the lysate was determined by bicinchoninic acid assay (BCA); 30 μ g of each protein sample was mixed with Laemmli sample buffer containing 5% β -mercaptoethanol and heated at 95°C for 5 min, followed by 10% SDS-PAGE under reducing conditions. The proteins were then electrophoretically transferred to a nitrocellulose membrane, followed by incubation with designated primary antibodies and secondary antibodies [1:3000, anti-rabbit (W4018) or anti-mouse IgG (W4028) from Promega, and anti-goat (sc-2020) from Santa Cruz]. The blots were detected by an enhanced chemiluminescence western blotting detection kit (Pierce).

Luciferase Assay

Three-week-old astrocytes were plated in 24-well plates and grown for 2–3 days to get to ~80% confluence prior to transfection with luciferase plasmid vectors overnight or in combination with expression vectors in some cases. pGL3 EAAT2Pro-954 was a generous gift from Dr. Paul B. Fisher (Virginia Commonwealth University, Richmond, VA) and pGL3 EAAT1 plasmid vector was a gift from Dr. David J. Volsky (Columbia University, New York, NY). ER- α and ER- β plasmids were kindly provided by Dr. Ken-Ichi Matsuda (Kyoto Prefectural University, Japan). A luciferase reporter vector for NF- κ B was from Clontech. Dominant negative constructs of I κ B α (I κ B α - N) and I κ B β (I κ B β - N) were from Dr. Dean Ballard (Vanderbilt University, Nashville, TN). After transfection with Lipofectamine 2000, cells were switched to the growth medium containing 5% FBS. The cells were treated with the designated compounds in Opti-MEM for the indicated time periods. Luciferase activity was measured with the Bright-Glo Luciferase kit (Promega) according to the manufacturer's instructions and normalized to the protein content as determined by Bradford protein assay (Bio-Rad). Normalization was verified by co-

transfecting with firefly reporter pGL4.75 plasmids (Promega) carrying the *Renilla* luciferase reporter gene.

Enzyme-linked Immunosorbent Assay (ELISA)

TNF- α release from astrocytes was determined by the rat TNF- α ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, the media samples (100 μ L/well) were collected from the 24-well plates after the designated periods of time of exposure with compounds, followed by ELISA. The optical density of each well was determined, using a microplate reader set to 450 nm with wavelength correction at 570 nm.

mRNA Isolation and Real-Time PCR

The total RNA was extracted using Trizol reagent and 2 μ g of RNA was transcribed using high capacity cDNA reverse transcription kit (Applied biosystems, USA). The quantitative real-time PCR was carried out in CFX96 real time PCR detection system (Bio-Rad) with the following sets of primers, forward: 5'-GGA AGA TGG GTG AAC AGG C-3' and reverse: 5'-TTC CCA CAA ATC AAG CAG G-3' for GLT-1, forward: 5'-TCC CTC AAG ATT GTC AGC AA-3' (GAPDH forward) and reverse 5'-AGA TCC ACA ACG GAT ACA TT-3' for GAPDH. The reactions were carried out in a total volume of 25 μ L, containing a 1 μ g of cDNA template of each sample, 0.4 μ M of the appropriate primers and RT2 SYBR Green qPCR Master Mix (SABiosciences/Qiagen). The PCR included one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s and at 60°C for 1 min. All samples were normalized relative to GAPDH. A web-based PCR array data analysis (SA Biosciences/Qiagen) was used to analyze the data.

Chromatin Immunoprecipitation (ChIP) Assays

ChIP analysis was performed with the EZ-ChIP chromatin immunoprecipitation kit (Millipore), following the manufacturer's instructions. Briefly, protein-DNA complexes were cross-linked with formaldehyde, followed by washing with ice cold PBS. The scraped cells were lysed with SDS lysis buffer and aliquots of the cell lysates were sonicated to lengths of 100–500 bp. Samples were diluted with ChIP dilution buffer and cleared with Protein G Sepharose. After saving a portion of the reaction sample for PCR amplification as inputs, the remainder was incubated overnight at 4°C with p65 or p50 NF- κ B antibodies (Santa Cruz), or control rabbit IgG (Millipore). The antibody containing complexes were eluted after series of washes with low salt and high salt buffers. Finally, DNA was purified using spin columns and PCR was carried out with the following primers for NF- κ B (–272, EAAT2 promoter), forward: 5'-CTG TGG GAC TCC CCA TCC CAG-3' and reverse: 5'-TGG CAG GAT CCC AGG GTC TAA-3'. PCR products were resolved on 1% agarose gel and visualized under UV light.

Glutamate Uptake Assay

The uptake of [3 H] glutamic acid was determined as previously described (Mutkus et al., 2005). Briefly, cells were treated with compounds in Opti-MEM for the indicated times and then washed twice with pre-warmed uptake buffer (122 mM NaCl, 3.3 mM KCl, 0.4 mM MgSO₄, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 25 mM HEPES, and 10 mM D-(+)-glucose, pH

7.4). The glutamate uptake assay was initiated by adding a mixture of non-radioactive glutamate and 0.25 $\mu\text{Ci/mL}$ L-[^3H] glutamate (specific activity, 49.0 Ci/mmol; PerkinElmer, Waltham, MA) to the reaction media to provide a final concentration of 100 nM glutamate. The reaction was continued for 10 min at 37°C and terminated by washing 3 times with ice-cold PBS, immediately followed by cell lysis in 1 mL of 1 N NaOH. An aliquot of 750 μL was taken out in scintillation vials and neutralized with 75 μL of 10 N HCl. After adding 5 mL of liquid scintillation fluid, the radioactivity was measured using a liquid scintillation counter (LS 6500; Beckman Coulter). The uptake activity was calculated as nmol glutamate/mg protein/min after correcting for protein levels.

Cell Viability Assay

Cells (2×10^4 /well) grown in 96-well plates were changed to Opti-MEM and treated with the designated compounds for 24 h. Then, 10 μL of MTT (5 mg/mL in PBS) was added to the cell cultures with the fresh medium and incubated for 4 h. The formazan crystals were dissolved in 100 μL of dimethyl sulfoxide and the absorbance was measured at 570 nm using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA).

Statistical Analysis

Data are expressed as a mean $\pm$ SEM. One-way analysis of variance 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; 'n' represents the statistical unit representing the number of cultures used (not wells). The number of samples for each experiment was at least three for western blots and real-time PCR analyses and four for glutamate uptake and luciferase assays. Data analyses were carried out with GraphPad software (Graph Pad, San Diego, CA).

Results

RX Enhances GLT-1 Expression and Function

We and others have previously reported that E2 and TX increase GLT-1 mRNA and protein levels (Lee et al., 2012b; Pawlak et al., 2005). In the present study, we sought to determine if RX, a SERM exerting less side effects compared to TX, also modulates astrocytic glutamate transporters. The results showed that 1 μM RX significantly ($p < 0.01$) increased the promoter activity of GLT-1 (we used EAAT2 promoter reporter vector, a human form of GLT-1 with >70% homology and similar characteristics to GLT-1) in astrocytes (Fig. 1A). The same concentration of RX also increased mRNA levels of GLT-1 in a time-dependent manner, reaching a plateau at 4 h (Fig. 1B), as well as GLT-1 protein expression (Fig. 1C). To determine whether the effect of RX on glutamate uptake was via GLT-1, we performed the glutamate uptake assay in the presence of UCPH-101 (20 μM), an inhibitor of GLAST. RX at 0.5 ($p < 0.05$) or 1 μM ($p < 0.001$), significantly increased glutamate uptake activity after 24 h incubation in astrocytes (dbcAMP was used as a positive control) (Fig. 1D).

RX Activates Multiple Signaling Pathways to Upregulate GLT-1

Several lines of studies have demonstrated that mechanism of E2-induced neuroprotection involves the activation of rapid signaling pathways such as MAPK/ERK and PI3K/Akt (Honda et al., 2000; Singer et al., 1999; Singh 2001; Singh et al., 2000; Zhang et al., 2001). We investigated if RX activates signaling pathways in astrocytes and the results showed that RX induced the phosphorylation of ERK, Akt, EGFR and CREB within 5 min (Fig. 2A). Accordingly, we applied specific inhibitors for these pathways to determine the role of each signaling protein in the action of RX on GLT-1 expression. The results showed that inhibition of the ERK pathway completely abolished RX-induced GLT-1 expression (Fig. 2B). Interestingly, RX phosphorylated Akt, but inhibition of this pathway did not abrogate the effect of RX on GLT-1 expression (Fig. 2A,B). Given that RX phosphorylated CREB and EGFR (Fig. 2A), we also determined if these signaling proteins modulated the effect of RX on GLT-1 expression. The specific inhibitors of these pathways, H89 (a PKA inhibitor to block CREB activation) and AG 1478 (AG, an EGFR inhibitor), abolished RX-induced GLT-1 expression and its promoter activity (Fig. 2C,D,E).

RX Upregulates GLT-1 via the NF- κ B Pathway at the Transcriptional Level

Since NF- κ B is the central pathway for the regulation of GLT-1 in response to positive stimuli (Su et al., 2003), we tested if NF- κ B plays a role in RX-induced upregulation of GLT-1. There are three NF- κ B binding sites in the GLT-1 promoter and mutation of these sites reduces GLT-1 promoter activity (Sitcheran et al., 2005). Our results showed that the enhancing effect of RX on GLT-1 promoter activity was completely abolished by PDTC, a pharmacological inhibitor of NF- κ B (Fig. 3A). RX also increased the NF- κ B reporter activity in astrocytes (Fig. 3B). Next, we determined if RX enhanced the binding of NF- κ B to the GLT-1 promoter. The ChIP analysis showed that RX induced the recruitment of NF- κ B p65 and p50 to the GLT-1 promoter (Fig. 3C). Further, RX-induced increase of GLT-1 protein levels was completely abrogated by PDTC (Fig. 3D).

RX Effect is Mediated by ER- α , ER- β and G-protein Coupled Receptor 30 (GPR30)

The neuroprotective effect of SERMs are mediated by activation of the nuclear ER receptors, ER- α and ER- β , as well as the G protein-coupled ER, GPR30 (Abdelhamid et al., 2011). Our results showed that inhibition of GPR30 with G15, a selective antagonist of GPR30, reduced GLT-1 expression and abolished RX-induced GLT-1 expression, indicating that GPR30 mediates the effect of RX on GLT-1 upregulation (Fig. 4A). With overexpression of ER- α or ER- β , RX further augmented RX-induced enhancement of GLT-1 promoter activity, GLT-1 protein expression and glutamate uptake (Fig. 4B–D). These results clearly indicate that these three ERs can mediate the effect of RX on GLT-1 expression.

Effects of ER- α/β Signaling on GLT-1 Expression is via Cellular Signaling Pathways such as ERK, Akt and EGFR

We previously reported that ER- α and ER- β enhanced GLT-1 expression (Lee et al., 2012b). Thus, we attempted to elucidate the molecular mechanisms involved in upregulation of GLT-1 expression. The results show that ICI 182,780 (ICI), a non-selective ER- α/β

antagonist, blocked the effects of DPN (a selective ER- β agonist) and PPT (a selective ER- α agonist) on GLT-1 expression (Fig. 5A). ICI alone increased GLT-1 expression significantly, possibly due to its agonistic effect on GPR30 (Lee et al., 2012a; Thomas et al., 2005). To further understand the intracellular signaling mechanisms of the ER- α/β pathways on GLT-1 expression, astrocytes were incubated with PPT or DPN in the presence of AG, PD and LY, which are pharmacological inhibitors of EGFR, ERK and Akt pathways, respectively. As shown in Fig. 5B and 5C, the inhibition of EGFR partially, but significantly reduced the effects of PPT and DPN on GLT-1 expression ($p < 0.05$), whereas the inhibition of ERK and Akt completely abrogated the effects of PPT and DPN on GLT-1 protein expression.

RX Attenuates Mn-induced Impairment of GLT-1

Mn is an essential element required for normal cellular processes, but its excessive accumulation in the brain causes neurotoxicity referred to as manganism (Bowman et al., 2011). Impairment of glutamate transporters has been postulated as one of the mechanisms involved in Mn-induced neurotoxicity, and both E2 and TX reverse Mn-induced impairment of glutamate transporters (Lee et al., 2009a). To determine if RX also mimics those effects, we tested the role of RX on the Mn-induced reduction of glutamate transporter GLT-1 expression and function. Pretreatment of astrocytes with 1 μ M RX for 18 h significantly attenuated Mn (250 μ M for 6 h)-induced reduction of GLT-1 promoter activity, protein expression and glutamate uptake (Fig. 6A–C). Moreover, we also tested if RX plays a role in cell survival against Mn-induced cell death. Mn (250 μ M) caused ~30% cell death after 24 h of exposure in astrocytes, but pretreatment with RX (1 μ M) for 24 h prior to Mn exposure completely blocked this Mn toxicity (Fig. 6D).

RX Attenuates Mn-induced TNF- α Production

To further understand the Mn action on GLT-1, we postulated that Mn-produced TNF- α might mediate Mn repression of GLT-1 since TNF- α is known to repress GLT-1 expression (Sitcheran et al., 2005). First, we confirmed the inhibitory effect of TNF- α on GLT-1 promoter activity (Fig. 7A) and then tested whether Mn induced TNF- α release in astrocytes by ELISA. Astrocytes treated with 250 and 500 μ M of Mn for 3 h showed a significant increase in TNF- α release into the media (Fig. 7B₁) and this effect was more prominent (3-fold) with a longer treatment period (24 h) at a lower Mn concentration (125 μ M) (Fig. 7B₂) that does not cause cell death (Lee et al., 2009b). Pretreatment with RX 1 μ M for 18 h prior to Mn exposure for 6 h significantly attenuated Mn-induced TNF- α production (Fig. 7C).

RX Upregulates GLAST Expression

In addition to GLT-1, GLAST (EAAT1 in human), another astrocytic glutamate transporter, plays a critical role in clearing the excessive glutamate from the synaptic clefts (Rosas et al., 2007). We tested if RX also modulates GLAST expression and function in astrocytes. Our results showed that RX significantly increased GLAST promoter activity and protein expression (Fig. 8A,B). Moreover, RX reversed Mn-induced reduction of GLAST protein (Fig. 8C). RX also increased glutamate uptake activity via GLAST in astrocytes in the presence of DHK, a selective GLT-1 antagonist (Kawahara et al., 2002) (Fig. 8D). In addition, to determine the specificity of RX effects on astrocytic glutamate transporters, we

tested if RX increased the expression of the neuronal glutamate transporter excitatory amino acid carrier 1 (EAAC1, EAAT3 in humans) which is mainly expressed in neurons, using the primary neuronal cultures from cortices of embryonic rat brain. The results showed that RX did not alter EAAC1 expression as well as glutamate uptake in primary neurons (Fig. 8E and F), indicating that the effects of RX are specific to the astrocytic glutamate transporters, GLT-1 and GLAST.

NF- κ B Mediates RX-induced GLAST Upregulation

To determine if NF- κ B mediates RX-induced upregulation of GLAST, we first determined the role of NF- κ B on GLAST expression as NF- κ B is a main transcription factor that regulates GLT-1. The overexpression of NF- κ B p65 and p50 subunits, respectively, led to a significant increase of GLAST promoter activity in astrocytes (Fig. 9A, B). Consistently, the overexpression of mutants of I κ B- α or I κ B- β , which inhibit the activation of NF- κ B, decreased the GLAST promoter activity (Fig. 9C). The RX-induced increases of GLAST promoter activity and protein levels were abrogated by PDTC, a pharmacological inhibitor of NF- κ B (Fig. 9D and 9E), indicating that NF- κ B plays a significant role in RX-induced upregulation of GLAST.

ER- α , ER- β and GPR30 mediate RX-induced upregulation of GLAST

Since all three ERs we tested mediated RX-induced GLT-1 upregulation (Fig. 4), we sought to determine if these ERs also modulate GLAST expression. The results revealed that RX significantly increased GLAST promoter activity and protein levels in astrocytes when ER- α or ER- β were overexpressed, respectively (Fig. 10A, B and C). Moreover, G15, a GPR30 antagonist, abrogated RX-enhanced GLAST protein expression (Fig. 10D). These results indicate that ER- α , ER- β and GPR30, mediate RX-enhanced GLAST expression.

Discussion

Our findings from the present study reveal that RX increased GLT-1 and GLST protein levels as well as glutamate uptake in astrocytes. RX-induced increase of GLT-1 expression was mediated by both the nuclear ERs (ER- α and ER- β) and G protein-coupled ER GPR30, through activation of multiple signaling proteins such as MAPK, EGFR, PKA, CREB and NF- κ B. RX also increased expression of another critical astrocytic glutamate transporter GLAST with similar mechanisms. RX did not modulate neuronal glutamate transporter EAAT3 (EAAC1), suggesting the RX effects are specific on astrocytic glutamate transporters, GLT1 and GLAST.

Impairment of astrocytic glutamate transporters has been associated with various neurodegenerative diseases including Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS). Therefore, drugs targeting astrocytic glutamate transporters to enhance their expressions and functions have been considered as potential therapeutics for these neurodegenerative disorders (Kim et al., 2011; Lin et al., 2012; Sheldon and Robinson, 2007). Several compounds have been reported to increase GLT-1 expression including ceftriaxone, phencyclidine and histone deacetylase inhibitors (Baltan et al., 2011; Fattorini et al., 2008; Kim et al., 2011; Lee et al., 2008), but no effective

therapeutics has been approved for its use as a neuroprotective agent. Interestingly, several SERMs including RX and TX have been shown to be neuroprotective in clinical settings as well as animal models of neurodegenerative diseases (Dhandapani and Brann, 2002). This suggests that SERMs may have potential to be developed into neuroSERMs if the precise molecular and cellular targets are identified. In line with this notion, our findings on RX-induced enhancement of glutamate transporters combined with its therapeutic effect on osteoporosis could provide invaluable information for the development of practical and effective neuroSERMs.

RX exerts neuroprotection against multiple toxicity models including oxidative stress, excitotoxicity and inflammation (Jayakumar et al., 2006; Sitcheran et al., 2005). For example, RX protects against oxidative stress in rat primary neuronal cultures (Abdelhamid et al., 2011) and kainic acid-induced excitotoxicity in hippocampus of ovariectomized rats (Ciriza et al., 2004; Yalcin et al., 2005). RX also exerts an anti-inflammatory effect in astrocytes by reducing lipopolysaccharide (LPS)-induced cytokines, interleukin-6 (IL-6) and interferon- γ -inducible protein-10 (IP-10) (Cerciat et al., 2010). Our findings also indicate that RX attenuates Mn-induced TNF- α production in astrocytes (Fig. 7). Oxidative stress and inflammation are closely associated with impairment of glutamate transporters (Jayakumar et al., 2006; Sitcheran et al., 2005). For example, TNF- α reduces GLT-1 promoter activity, mRNA and protein levels in H4 glioma cells as well as primary astrocytes (Karki et al., 2014; Sitcheran et al., 2005), suggesting that TNF- α may play a central role by cross-talking between inflammation and excitotoxicity.

RX activates multiple intracellular signaling proteins in various cell types (Abdelhamid et al., 2011; Dai et al., 2012; Du et al., 2004; Fang et al., 2013; Lee et al., 2011). These signaling pathways also play a role in RX-induced GLT-1 upregulation (Fig. 2). Interestingly, RX phosphorylated Akt, but the RX-enhancing effect on GLT-1 expression did not require Akt activation (Fig. 2B). In contrast, RX has been shown to protect against hypoxic cell injury by the activation of the Akt pathway (Stice et al., 2012) suggesting that the role of Akt in RX-induced cellular events is variable. On the other hand, the PI3K/Akt pathway played a critical role in mediating the effects of ER- α , ER- β and GPR30 on GLT-1 expression [Fig. 4, (Lee et al., 2012a)]. These results suggest that multiple signaling pathways, including the ER-independent pathway, are involved in the action of RX on GLT-1 expression.

The present results also indicate that the NF- κ B pathway plays a critical role in RX-induced enhancement of GLT-1 expression at the transcriptional level. The role of NF- κ B in GLT-1 expression has been previously demonstrated in GLT-1 upregulation by epidermal growth factor (EGF) and dbcAMP (Su et al., 2003), G1 (Lee et al., 2012a), TX (Karki et al., 2013) and rapamycin in astrocytes (Ji et al., 2013). NF- κ B subunits p65 and p50 are involved in RX-enhanced GLT-1 expression, albeit NF- κ B p65 bound to the GLT-1 promoter with a higher efficiency than that of p50. These data indicate that p65 is a dominant NF- κ B subunit recruited by RX in GLT-1 upregulation. Taken together, NF- κ B plays as a critical transcriptional activator in RX-induced upregulation of GLT-1.

Based on our results, RX exerts an agonistic effect on the ERs in astrocytes for GLT-1 expression, which could be an important pharmacological feature of RX mechanism for neuroprotection. RX activated ER- α with a higher efficacy than ER- β to increase GLT-1 promoter activity, indicating that RX preferentially activates ER- α over ER- β to mediate its effect on GLT-1 expression. GPR30 also plays a significant role in RX-induced upregulation of GLT-1 since inhibition of GPR30 with its selective antagonist G15 reduced the basal levels of GLT-1 protein, and significantly inhibited RX-induced GLT-1 expression (Fig. 4). GPR30 has also been shown to mediate the effects of E2 and SERMs on neuroprotection (Abdelhamid et al., 2011; Lee et al., 2012a).

Since RX activated multiple ER subtypes, we further determined the role of each ER in GLT-1 expression and its associated cellular signaling mechanisms using selective agonists for ER subtypes. The nuclear ERs, ER- α and ER- β , are involved in upregulation of GLT-1 as these effects were blocked by ICI, a non-selective antagonist of ER- α and ER- β (Fig. 5A). The G protein-coupled ER GPR30 also increases GLT-1 expression via the non-genomic ER pathways using multiple signaling pathways including ERK/MAPK, CREB and NF- κ B (Lee et al., 2012a). Although the nuclear ERs have been thought to induce estrogenic effects via the genomic pathway, numerous recent studies provide evidence that ER- α and ER- β also activate the non-genomic intracellular signaling pathways (Srivastava et al., 2011). The GLT-1 promoter does not contain an estrogen response element (ERE), and thus, both ER- α and ER- β modulate GLT-1 expression via the non-genomic membrane-associated ER- α/β , activating intracellular signaling pathways. This notion is supported by the results that the effects of ER- α and ER- β on GLT-1 expression were mediated by EGFR, ERK and Akt pathways (Fig. 5B,C). It has been reported that both ER- α and ER- β induce neuroprotection against glutamate excitotoxicity in hippocampal neurons via the non-genomic ER mechanisms (Zhao et al., 2004).

To mimic the pathological condition of neurodegeneration with impairment of GLT-1, we used Mn which induces a reduction of GLT-1 expression and glutamate uptake in astrocytes (Lee et al., 2012b) as well as manganism, PD-like pathology and impairment of GLT-1 in animals (Bowman et al., 2011; Erikson et al., 2007). Although E2 and TX also reverse the Mn repression of glutamate transporters (Lee et al., 2009a), the clinical advantage of RX over other SERMs or E2, exerting less peripheral side effects, should be underscored for developing neuroprotective therapeutics.

RX also increases the expression and function of GLAST, another critical astrocytic glutamate transporter and reverses Mn-induced reduction of GLAST, indicating that RX effect is not restricted to GLT-1 in astrocytes. The efficacy of RX-induced increase of glutamate uptake via GLT-1 was greater than those via GLAST (~2.3 fold via GLT-1 vs. ~1.4 fold via GLAST), suggesting that GLT-1 is the main target of RX in enhancing glutamate uptake activity. The mechanisms by which RX induces upregulation of GLAST are similar to those of GLT-1, utilizing the ERs and NF- κ B. The role of the NF- κ B pathway has been reported in amitriptyline/morphine-induced upregulation of GLAST in rat brain (Tai et al., 2008). The results also revealed that RX selectively targets the astrocytic glutamate transporters (GLAST and GLT-1) because it did not affect the neuronal glutamate transporter EAAT3 expression and function in primary neuronal cultures (Fig. 8E and F).

Taken together, the proposed mechanisms of RX action on GLT-1/GLAST are illustrated in Fig. 11. RX induces upregulation of GLT-1 and GLAST via ER- α , ER- β and GPR30, and subsequent activation of multiple signaling pathways including MAPK, EGFR, PKA-CREB and NF- κ B. Our results for RX-enhancing effects on astrocytic glutamate transporters provide potential for developing perfect neuro-SERMs which exert beneficial effects only in the brain without inducing undesirable peripheral side effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

RX enhances GLT-1 expression and function. **(A)** After overnight transfection with 0.5 μ g of the GLT-1 promoter reporter vector (pGL3-EAAT2Pro-954 luciferase vector), astrocytes were treated with RX (1 μ M, 24 h) followed by luciferase assay. **(B)** Astrocytes were treated with 1 μ M of RX for the indicated time points, with subsequent RT and qPCR assays. The levels of GLT-1 mRNA were normalized to GAPDH. **(C)** Astrocytes were treated with RX (0, 0.25, 0.5 and 1 μ M) and subjected to subsequent western blot analysis with quantification (lower panel) using β -actin as a loading control. **(D)** Glutamate uptake assay was performed after astrocytes were treated with RX in the presence of UCPH-101 (20 μ M, a GLAST inhibitor). dbcAMP was used as a positive control. * p <0.05, ** p <0.01, *** p <0.001, compared to controls determined by ANOVA followed by Tukey's *post hoc* test; N=4).

FIGURE 2.

Activation of MAPK, PKA and EGFR is required for RX-induced upregulation of GLT-1.

(A) Astrocytes were treated with 1 μ M of RX for the indicated time points, followed by western blot analysis to detect phospho- and total- ERK, Akt, CREB and EGFR proteins.

(B) Astrocytes were preincubated with PD98059 (50 μ M) or LY294002 (20 μ M) for 30 min prior to RX treatment for 24 h, followed by western blot analysis.

(C, E) After overnight transfection with the GLT-1 (EAAT2) promoter vector, astrocytes were preincubated with H89 (10 μ M) or AG1478 (10 μ M) for 30 min prior to RX (1 μ M) treatment for 24 h, followed by luciferase assay.

(D, F) Astrocytes were preincubated with H89 (10 μ M) or AG 1478 (10 μ M) for 30 min prior to RX (1 μ M) treatment for 24 h, followed by western blot analysis. The amount of GLT-1 protein expression was quantified for B, D and F (lower panel for B and D; right panel for F). (* p < 0.05, ** p < 0.01, *** p < 0.001, compared to the control; ANOVA followed by Tukey's *post hoc* test; N=4).

FIGURE 3.

NF-κB mediates RX-induced upregulation of GLT-1. **(A)** After overnight transfection with the GLT-1 (EAAT2) promoter vector, astrocytes were preincubated with PDTC (50 μM) for 30 min prior to RX (1 μM) treatment, followed by luciferase assay. **(B)** After overnight transfection with 0.5 μg of the NF-κB reporter vector overnight, astrocytes were treated with RX (1 μM) for 24 h, followed by luciferase assay. **(C)** The effect of RX on NF-κB p65 and p50 binding to the GLT-1 promoter regions was determined by ChIP assay; the final PCR products of the promoter sequences bound to p65 or p50 were quantified, and compared to controls (lower panels). **(D)** Astrocytes were preincubated with PDTC (50 μM) for 30 min prior to the addition of RX (1 μM) for 24 h and subsequent western blot analysis with quantification (right panel). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the control; ANOVA followed by Tukey's *post hoc* test; N=4).

FIGURE 4.

ER-α, ER-β and GPR30 mediate RX-induced upregulation of GLT-1. (A) Astrocytes were treated with RX (1 μM), G15 (1 μM) or RX plus G15 for 24 h, followed by western blot analysis to measure GLT-1 protein levels that are quantified in the lower panel. (B, C, D) After overnight co-transfection with 0.5 μg of the GLT-1 (EAAT2) promoter vector and 0.1 μg of either ER-α or β overexpression vectors, (B) astrocytes were treated with RX (1 μM) for 24 h, followed by luciferase assay, (C) GLT-1 protein expression with quantification (right panel) and (D) glutamate uptake assay. (# $p<0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, @ $p<0.05$, compared to the control; ANOVA followed by Tukey's *post hoc* test; N=4).

FIGURE 5.

Both ER- α and ER- β induce GLT-1 expression via multiple signaling pathways. (A) Astrocytes were treated with PPT (200 pM) or DPN (200 pM) alone, or with ICI (1 μ M) for 24 h. GLT-1 protein expression level (right panel) was determined by western blot analysis. (B) Astrocytes were preincubated with AG 1478 (1 μ M), an EGFR inhibitor, for 30 min prior to treatment with PPT (200 pM) or DPN (200 pM) for 24 h, followed by western blot analysis to determine GLT-1 protein expression. (C) Astrocytes were preincubated with 50 μ M of PD (ERK inhibitor) or 20 μ M of LY (PI3K inhibitor) for 30 min prior to treatment with PPT (200 pM) or DPN (200 pM) for 24 h, followed by western blot analysis with quantification. (***) $p < 0.001$, * $p < 0.05$, compared to the control; @ significantly different at $p < 0.05$; ANOVA followed by Tukey's *post hoc* test; N=4).

FIGURE 6.

RX reverses Mn-induced reduction of GLT-1 expression and glutamate uptake. (A) After overnight transfection with the GLT-1 (EAAT2) promoter vector, astrocytes were pretreated with RX (1 μ M) for 18 h prior to Mn (250 μ M) exposure for 6 h, followed by luciferase assay. (B, C) Astrocytes were pretreated with RX (1 μ M) for 18 h prior to Mn (250 μ M) exposure for 6 h, followed by (B) western blot analysis with quantification (lower panel) and (C) glutamate uptake assay. (D) Astrocytes were pretreated with RX (1 μ M) for 24 h and the subsequent exposure to Mn (250 μ M) for 24 h, followed by the MTT assay.

(# p <0.05, * p <0.05 ** p <0.01, compared to the control; ANOVA followed by Tukey's *post hoc* test; N=4).

FIGURE 7.

RX attenuates Mn-induced TNF- α release which reduces GLT-1 (EAAT2) promoter activity in astrocytes. **(A)** After overnight transfection with the EAAT2 promoter vector, astrocytes were treated with TNF- α (15 ng/mL) for 6 h, followed by luciferase assay. **(B)** Astrocytes were treated with Mn at either **(B₁)** 250 and 500 μ M for 3h or **(B₂)** 125 μ M for 24 h, followed by the collection of media and subsequent ELISA. **(C)** Astrocytes were pretreated with RX (1 μ M) for 18 h prior to Mn (250 μ M) exposure for 6 h, followed by ELISA. ($\#p<0.05$, $##p<0.01$, $*p<0.05$, $***p<0.001$, compared to the control; Student's t-test; N=3).

FIGURE 8.

RX increases GLAST expression and reverses Mn-induced reduction of GLAST protein.

(A) After overnight transfection with the GLAST (EAAT1) promoter vector, astrocytes were treated with RX (1 μM) for 24 h, followed by luciferase assay. (B) Astrocytes were treated with RX (0, 0.25, 0.5 and 1 μM) for 24 h and subsequent western blot analysis to measure GLAST protein levels. (C) Astrocytes were pretreated with RX (1 μM) for 18 h prior to Mn (250 μM) exposure for 6 h, followed by western blot analysis to measure GLAST protein levels. (D) Astrocytes were treated with RX (1 μM) in the presence of DHK (100 μM, a GLT-1 inhibitor), followed by a glutamate uptake assay. (E) Primary neuronal cultures from the rat brain were treated with RX (1 μM) for 24 h, followed by western blot analysis. (F) Glutamate uptake activity was measured in primary neurons after RX (1 μM) treatment for 24 h. (## $p < 0.01$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared to the control; ANOVA followed by Tukey's *post hoc* test; N=4).

FIGURE 9.

NF-κB mediates RX-induced upregulation of GLAST. (**A**, **B**) Astrocytes were co-transfected with 0.5 μg of the GLAST (EAAT1) promoter vector and either (**A**) 0.1 μg of p65 or its empty vector pRC/RSV as a control or (**B**) p50 or its empty vector pcDNA as a control, respectively, followed by luciferase assay. (**C**) Astrocytes were co-transfected with the GLAST (EAAT1) promoter vector and dominant-negative mutant NF-κB inhibitors, IκBα-N and IκBβ-N (pCMV4 as a control) followed by luciferase assay. (**D**) After overnight transfection with the GLAST (EAAT1) promoter vector, astrocytes were preincubated with 50 μM of PDTC for 30 min prior to RX (1 μM) treatment for 24 h, followed by luciferase assay. (**E**) Astrocytes were preincubated with PDTC prior to RX (1 μM) treatment for 24 h and subsequent western blot analysis to determine GLAST protein levels with quantification (lower panel). (@ $p < 0.05$, ## $p < 0.01$, *** $p < 0.001$, compared to the control; ANOVA followed by Tukey's *post hoc* test; N=4).

FIGURE 10.

ER- α , ER- β and GPR30 mediate RX-induced upregulation of GLAST. (**A, B, C**) Astrocytes were transfected overnight with 0.5 μ g of GLAST (EAAT1) promoter vector and 0.1 μ g of either ER- α or ER- β overexpression vectors followed by RX (1 μ M) treatment for 24 h. Then, GLAST (EAAT1) promoter activity was determined by luciferase assay (**A** and **B**) and western blot analysis was performed to measure GLAST protein levels that are quantified in the lower panel (**C**). (**D**) Astrocytes were treated with RX (1 μ M), G15 (1 μ M) or RX plus G15 for 24 h, followed by Western blot analysis to measure GLAST protein levels with quantification in the lower panel. ** $p < 0.01$, compared to the control; ANOVA followed by Tukey's *post hoc* test; N=4).

FIGURE 11.

Proposed mechanisms of RX-induced increase of glutamate transporters. RX activates both nuclear ERs (ER- α /ER- β) and GPR30 leading to activation of MAPK, CREB, EGFR and NF- κ B signaling pathways, resulting in upregulation of GLT-1 and GLAST. This leads to enhanced glutamate uptake and ultimately, protection of neurons from glutamate-induced excitotoxicity.