GPR30 Regulates Glutamate Transporter GLT-1 Expression in Rat Primary Astrocytes

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Background: Estrogen increases glutamate transporter GLT-1 expression.

Results: G1, a selective agonist of GPR30, significantly increased GLT-1 expression in rat primary astrocytes, and GPR30 suppression reduced GLT-1 expression.

Conclusion: The G protein-coupled estrogen receptor GPR30 plays a critical role in the regulation of GLT-1 expression.

Significance: Understanding the mechanism of GPR30 regulation on GLT-1 expression is important to develop therapeutics against excitatory neuronal injury.

The G protein-coupled estrogen receptor GPR30 contributes to the neuroprotective effects of 17β-estradiol (E2); however, the mechanisms associated with this protection have yet to be elucidated. Given that E2 increases astrocytic expression of glutamate transporter-1 (GLT-1), which would prevent excitotoxic-induced neuronal death, we proposed that GPR30 mediates E2 action on GLT-1 expression. To investigate this hypothesis, we examined the effects of G1, a selective agonist of GPR30, and GPR30 siRNA on astrocytic GLT-1 expression, as well as glutamate uptake in rat primary astrocytes, and explored potential signaling pathways linking GPR30 to GLT-1. G1 increased GLT-1 protein and mRNA levels, subject to regulation by both MAPK and PI3K signaling. Inhibition of TGF-α receptor suppressed the G1-induced increase in GLT-1 expression. Silencing GPR30 reduced the expression of both GLT-1 and TGF-α and abrogated the G1-induced increase in GLT-1 expression. Moreover, the G1-induced increase in GLT-1 protein expression was abolished by a protein kinase A inhibitor and an NF-κB inhibitor. G1 also enhanced cAMP response element-binding protein (CREB), as well as both NF-κB p50 and NF-κB p65 binding to the GLT-1 promoter. Finally, to model dysfunction of glutamate transporters, manganese was used, and G1 was found to attenuate manganese-induced impairment in GLT-1 protein expression and glutamate uptake. Taken together, the present data demonstrate that activation of GPR30 increases GLT-1 expression via multiple pathways, suggesting that GPR30 is worthwhile as a potential target to be explored for developing therapeutics of excitotoxic neuronal injury.

The female sex hormone, 17β-estradiol (E2), not only modulates sexual differentiation, endocrine regulation, and initiation of reproductive behavior (1) but also brain maturation, neuron morphology, and synaptic plasticity (2). In addition, E2 exerts neuroprotective effects in various in vitro and in vivo models (3, 4). The mechanism(s) that mediate these neuroprotective effects have yet to be fully elucidated.

The conventional effects of E2 are mediated by its nuclear receptors, estrogen receptor (ER)-α and ER-β, which act as ligand-activated transcription factors (5). This ligand-receptor complex binds to estrogen response elements in the promoter regions of genes regulated by E2. In general, the classical effects of E2 take hours to days to modulate gene expression. However, recent studies have established that E2 can also mediate rapid physiological events (seconds to minutes), such as the activation of signaling pathways (6) and ion channels (7), events that cannot be explained by classical E2 signaling via the nuclear ER-α and ER-β.

GPR30, a G protein-coupled receptor, mediates the biological effects of E2, predominantly via membrane-associated activation of intracellular signaling pathways linked to growth factor receptors (8). Upon binding to GPR30, E2 transactivates EGF receptor (EGFR) followed by ERK1/2 activation (6). GPR30 up-regulates nerve growth factor and Bcl-2 (9, 10) and promotes neuronal calcium release (11). The functions of GPR30 have been largely studied with its selective ligand, G1 (12), establishing the role of GPR30 in transcriptional regulation of genes involved in cell proliferation and tumorigenesis (13).

2 The abbreviations used are: E2, 17β-estradiol; PP2, 3-(4-chlorophenyl)-1-((1,1-dimethyl-1H-pyrazolo|3,4-d|pyrimidin-4-amine); H89, N-[2-[[3-(4-bromophenyl)-2-propenyl]amino(ethyl)-5-isooquinolinesulfonamide dihydrochloride; G15, (3aS,4R*,9bR*)-4-((6-bromo-1,3-benzodioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl)-ethanone; GLT-1, glutamate transporter-1; EGFR, EGF receptor; GLAST, glutamate aspartate transporter; MEM, minimum essential medium; ANOVA, analysis of variance; PT, pertussis toxin; ER, estrogen receptor; CREB, cAMP response element-binding protein; Mn, manganese.

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GPR30 Regulation of GLT-1

Although E2 is neuroprotective in various experimental and clinical studies (14), its long term clinical utility is hampered by elevated risk for breast and uterine cancers because of its action on genomic nuclear ERs. Thus, development of alternative therapeutics has been of intense interest. Selective estrogen receptor modulators, such as tamoxifen and raloxifene, have shown neuroprotection in various experimental models (14). One candidate is G1, which selectively activates GPR30, thus avoiding these risks; GPR30 has also been implicated in neuroprotection in several experimental models, promoting neuron survival upon global ischemia (15), oxygen-glucose deprivation (16), and oxidative stress (17).

Within the brain, astrocytes are the major cellular target of E2 (18). E2 regulates expression of neuroprotective glial proteins, particularly heat shock proteins (Hsp) (19, 20), and the glutamate transporters GLT-1 and glutamate aspartate transporter (GLAST) (21–23), as well as a variety of growth factors, including TGF-α (24), TGF-β1 (25), basic FGF (25), and insulin-like growth factor I (26, 27).

Because GLT-1 plays an essential role in removing excess extracellular glutamate (28), impairment in GLT-1 function is associated with multiple neurodegenerative diseases, including amyotrophic lateral sclerosis, Parkinson disease, and Alzheimer disease (29). Therefore, enhancing GLT-1 expression may offer a pertinent strategy for the treatment and prevention of neurodegeneration accompanying excitotoxic insults.

Although the gene for GLT-1 has been identified, the promoter elements of GLT-1 (EAAT2 in human) have not been fully characterized, and the mechanisms regulating their expression remain to be established. EGFR agonists have been implicated in the regulation of GLT-1 expression (30). Neuronal regulation of GLT-1 by neuron-secreting factors and neuronal contacts were also explored (31, 32). Given that GPR30 transactivates the EGFR pathway (33) and E2 enhances glutamate transporter expression (21, 34), we hypothesized that E2 and selective estrogen receptor modulators promote neuronal survival via the GPR30-EGFR/GLT-1 pathway. Therefore, we studied the effect of G1 (a selective agonist of GPR30) on GLT-1 expression and explored the mechanism by which G1/GPR30 regulates GLT-1 expression in rat primary astrocytes.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents, media, and sera were purchased from Invitrogen. [3H]Glutamic acid was purchased from Amersham Biosciences. PD98059, LY294002, PP2, tyrphostin AG1478 (AG1478), pyrrolidine dithiocarbamate, H89, PD98059, LY294002, PP2, tyrphostin AG1478 (AG1478), pyrrolidine dithiocarbamate, H89, from Amersham Biosciences. PD98059, LY294002, PP2, tyrphostin AG1478 (AG1478), pyrrolidine dithiocarbamate, H89, from Amersham Biosciences.

Cell Culture—To prepare primary astrocytes, following careful removal of meninges, the cerebral hemispheres of 1-day-old newborn rats (Sprague-Dawley) were dissociated using Dispase II (Boehringer-Mannheim Biochemicals; neutral protease, Dispase Grade II). Twenty-four hours after the initial plating, the media were changed to preserve the adhering astrocytes and to remove neurons and oligodendrocytes. The cultures were maintained at 37 °C in a 95% air, 5% CO2 incubator for 3 weeks in MEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. These cultures showed >95% positive staining for glial fibrillary acidic protein, an astrocyte-specific marker. All of the experiments were performed 3 weeks after isolation.

Glutamate Uptake Assay—Uptake of [3H]glutamic acid was measured as described previously (22, 39). In brief, medium was removed, and the cells were washed rapidly twice with Opti-MEM before treatment with compounds in Opti-MEM for the indicated times at 37 °C. The cells were then washed twice with prewarmed 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). Uptake was initiated by aspirating the medium and adding prewarmed uptake buffer containing 0.25 μCi/ml L-[3H]glutamate (specific activity, 49.0 Ci/mmol; Amersham Biosciences) at a final concentration of 100 μM (35). Uptake reaction was continued for 10 min at 37 °C and terminated by three washes with ice-cold PBS, immediately followed by cell lysis in 1 ml of 1 N NaOH. An aliquot of 750 μl was neutralized in 75 μl of 10 N HCl, and radioactivity was determined by liquid scintillation counter (LS 6500; Beckman Coulter) and corrected for protein levels to yield nmol glutamate/mg protein/min.

Western Blot Analysis—Astrocytes were treated with the designated compounds for the indicated periods, followed by two washes with cold Hank’s buffered salt solution. Subsequently, the cells were lysed with radio immunoprecipitation assay buffer, containing a protease inhibitor mixture (10 μM in 1 ml of lysis buffer; Sigma-Aldrich) as described previously (22). Lysate protein content was measured by bichinonic acid assay, and samples of 30 μg of protein were mixed with Laemmli sample buffer containing 5% β-mercaptoethanol and heated at 95 °C for 5 min, followed by SDS-PAGE under reducing conditions. The proteins were then electrophoretically transferred to a nitrocellulose membrane (Whatman). Western blot analysis was performed using primary antibodies (GLT-1, 1:1000, Millipore or Santa Cruz Biotechnology; β-actin, 1:5000, Sigma-Aldrich), phospho-ERK1/2 (1:1000), ERK1/2 (1:1000), phospho-Akt (Ser308; 1:1000), Akt (1:1000), phospho-EGFR (Tyr; 1:500), EGFR (1:1000), phospho-CREB (1:500), and CREB (1:1000), followed by secondary antibodies (1:3000, anti-guinea pig, rabbit, anti-mouse IgG peroxidase conjugates; Santa Cruz Biotechnology). The immunoreactive proteins were detected by an enhanced chemiluminescence Western blotting detection kit (Pierce). A monoclonal band of GLT-1 (70 kDa) was used to quantify the data. The intensities of all protein bands were normalized with the housekeeping gene, β-actin.

RT-PCR and Real Time PCR—Total RNA was extracted from cells using the RNeasy mini RNA isolation kit (Qiagen). Two μg of total RNA was transcribed with poly(dT) oligonucleotides.
and reverse transcriptase. PCR targeting GLT-1 and TGF-α was performed with Supermix (Invitrogen) in a final volume of 30 μl. Sequences of the primers were: 5′-CCT CAT GAG GAT GCT GAA GA-3′ (GLT-1 forward) and 5′-TCC AGG AAG GCA TCC AGG CTG-3′ (GLT-1 reverse); 5′-ACG GTC ACT GCT GTC ATT G-3′ (GLAST forward) and 5′-TGT GAC GAG ACT GCT GTC ATT G-3′ (GLAST reverse); 5′-TGG AGA ACA GCA CCT CCT CC-3′ (TGF-α forward) and 5′-TGG TGG ACT GCT GTC ATT G-3′ (TGF-α reverse); and 5′-TCC AGC ACG ATC TCC TAG TGG-3′ (GAPDH forward) and 5′-TGG AGA ACA TCC ACA ACG GAT ACA TT-3′ (GAPDH reverse). After 30 cycles of amplification (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), the samples were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. GAPDH was used as internal standard. The CFX96 real time PCR detection system (Bio-Rad) was used to amplify GLT-1 and GLAST. The reactions were carried out in a total volume of 25 μl, containing a 1 μg of cDNA template of each sample, 0.4 μM of the appropriate primers and RT2 SYBR Green qPCR Master Mix (SABiosciences/Qiagen). The PCR protocol consisted of one cycle at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. Each sample was normalized relative to GAPDH. The data were analyzed using a web-based PCR array data analysis (SABiosciences/Qiagen).

**Immunohistochemistry**—After treatment, the astrocytes were washed twice with TBS and fixed with 100% methanol for 10 min at room temperature. After rinsing in TBS (50 mM Tris, 150 mM NaCl, pH 7.4), the cells were incubated for 30 min at room temperature with TGT (TBS containing 5% normal goat serum and 0.1% Triton X-100) overnight at 4 °C in TGT containing rabbit polyclonal anti-GLT-1 (1:200; Santa Cruz Biotechnology), rinsed in TBS with 0.1% Triton X-100, and incubated for 2 h at room temperature in TGT containing anti-mouse IgG-fluorescein conjugates (1:200; Santa Cruz Biotechnology, Santa Cruz, CA). In separate experiments, primary or secondary antibodies were omitted to control for possible cross-reactivity. The cultures were covered with Vector Shield.
mounting medium (Vector Laboratories) and observed under a Nikon confocal microscope (A1R laser scanning confocal).

**siRNA Synthesis and Transfection**—Templates for siRNA synthesis were designed on the basis of the GPR30 sequences in which the total length was 21 bases and the relative GC content of the template was 55%. The sequences of GPR30 used were antisense 5'-H11032-CCU GGA CGA GCA GUA UUA CTT-3' and sense 5'-H11032-GUA AUA CUG CUC GUC GTT-3'. These target sequences were previously reported (31). Astrocytes were transfected for 3 days, followed by 8 h of treatment with G1 (2 μM) and measurement of mRNA expression.

**Transient Transfection and Luciferase Assay**—Astrocytes cultured in 500 μl of growth medium in 24-well plates for 2–3 days were transfected overnight with 0.5 μg of pGL3 basic reporter vector, containing EAA2Pro-954 (kindly provided by Dr. Fisher, Virginia Commonwealth University) and Lipo-fectamine 2000 transfection reagent (Invitrogen) in MEM containing 5% FBS. Dominant-negative cAMP response element-binding protein (A-CREB) expression vector was from Dr.
Charles Vinson (National Cancer Institute) and luciferase reporter vectors for NF-κB and CRE were from Clontech. The cells were then treated with the designated compounds in opti-MEM containing 5% FBS for 24 h. 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). In all experiments, normalization was verified by co-transfecting with firefly reporter pGL4.75 plasmids (Promega) carrying the Renilla luciferase reporter gene.

**Mutagenesis**—The GLT-1 (EAAT2) promoter (−954 to +44 bp) subcloned into pGL3 basic plasmid vector (36) was used. NF-κB triple mutant (−583/−272/−251) was a gift from Dr. Sitcheran (Texas A & M Health Sciences Center). To generate a mutation at the −308 CREB binding sequence, the sequence 5′-GCC CGG CGC GGG TGA TGT CTC TCG ACG AAA-3′ was changed to 5′-GCC CGG CGC GGG TGA TGT CTC TCG ACG AAA-3′ by PCR-based mutagenesis using a site-directed mutagenesis kit (Stratagene). The wild-type CACGCG (NheI) site was changed to CCCGGG (SmaI) using 5′-GCT CTT ACG CGT GCC AGC CCG GCC TCG AGA TC-3′ to identify mutant clones. Clones were sequenced to confirm mutations.

**Chromatin Immunoprecipitation 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 (10 min at room temperature) and sonicated to lengths of 100−500 bp; 100 μl of supernatant was mixed with 900 μl of ChIP dilution buffer (Millipore). After preclaring, 1% of the reaction was saved for PCR amplification as input data. The remainder was incubated overnight at 4 °C with the CREB antibody (Cell Signaling), p50 (Cell Signaling), p65 (Santa Cruz), or control rabbit IgG (Millipore). After isolation and washing of antibody-containing complexes, DNA was extracted. PCRs were performed using the following primers: for CREB, forward: 5′-GGG ACA ACA GAA GAG GGA CA-3′ and reverse: 5′-AGG GAT TGC AAG GTT TAG CC-3′ (−563 to −403); for NF-κB, forward: 5′-CTG TGG GAC TCC CCA TGG CAG GAT CCC AGG GTC TAA-3′. Products were resolved on 1% agarose gel and visualized under UV light.

**Statistical Analysis**—The mean and S.E. were determined for each set of data. One-way analysis of variance (ANOVA) followed by Tukey post hoc test was used to determine significance.

**RESULTS**

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GPR30 Activation Increases Astrocytic GLT-1 Protein and mRNA, as well as Glutamate Uptake—Astrocytes express both GPR30 protein and mRNA (37). Glutamate uptake in the astrocyte occurred via both GLAST and GLT-1. The G1-induced
increase in glutamate uptake was predominantly mediated via GLT-1 (supplemental Fig. S1). To understand the detailed mechanism for GPR30 enhancement on GLT-1 expression, astrocytes were treated for 24 h with G1 (0–4 μM), and net glutamate uptake was assayed (after 10 min of incubation with [3H]glutamate). G1 increased glutamate uptake in a concentration-dependent manner (Fig. 1A) concomitant with increased GLT-1 protein (Fig. 1B) and mRNA levels (Fig. 1C). GLT-1 protein forms a trimer (220 kDa) as well as a monomer (70 kDa) (Fig. 1B). Herein, the monomer was used for the analysis of band density. G1 also increased the expression of astrocytic GLAST protein and mRNA, but the effect on GLAST was less pronounced (Fig. 1D). The effects of G1 were specific for the glutamate transporters because it did not alter GAP43 protein levels (Fig. 1E, used as an example of brain protein). Compared with GLAST protein expression, the effect of G1 on GLT-1 expression was more pronounced. Comparison on protein expression levels of GLT-1, GLAST, and GAP43 are shown in Fig. 1F.

GPR30 Silencing with GPR30 siRNA Reduces Expression of GLT-1 and TGF-α—To determine whether GPR30 regulates GLT-1 expression, GPR30 was knocked down by siRNA transfection. Astrocytes were transfected with GPR30 siRNA for 3 days, followed by mRNA expression analysis by RT-PCR. Transfected cells were also treated with G1 for 8 h prior to mRNA analysis. As shown in Fig. 2A, GPR30 mRNA levels were significantly suppressed (p < 0.01) by siRNA, in turn, resulting in the repression of both GLT-1 and TGF-α mRNA expression compared with the scrambled RNA control groups (p < 0.01 and p < 0.05, respectively). The effect of G1 on GLT-1 expression was also abrogated in GPR30 knockdown astrocytes (Fig. 2B). Moreover, G15, a selective pharmacological antagonist of GPR30, completely suppressed G1-induced increase in GLT-1 protein expression (Fig. 2C). Compared with GLT-1, the effect of G1 on GLAST was less pronounced (data not shown).

GPR30 Activation Regulates Astrocytic GLT-1 Expression via the G Protein-coupled GPR30 Pathway—Because GPR30 is a G protein-coupled receptor, next we tested whether astrocytic GLT-1 expression is regulated by the G protein-mediated signaling pathway. As shown in Fig. 3A, pertussis toxin (PT), a G protein inhibitor, blocked both the G1-induced increase in astrocytic glutamate uptake as well as GLT-1 protein expression. PT only partially blocked the E2-induced increase in GLT-1 expression (Fig. 3B). These data establish that E2 enhances GLT-1 function not only via GPR30 signaling but also via other ERs, such as ER-α and ER-β. The effect of G1 on GLAST was similar to that of GLT-1 (Fig. 3C).

GPR30 Activation Regulates Astrocytic GLT-1 Expression via the MAPK/ERK, PI3K/Akt, TGF-α/EGFR, and Src Pathways—Because E2 increases phosphorylation of astrocytic MAPK and PI3K (34), we investigated whether these signaling pathways mediate the effect of G1 on GLT-1 expression. G1 activated MAPK/ERK as early as 5 min and PI3K/Akt by 15 min (Fig. 4A). Within 15 min of its addition to the media, G1 also activated EGFR (Fig. 4A). The MAPK/ERK inhibitor PD98059, the PI3K/Akt inhibitor LY294002, and the EGFR inhibitor AG1478 completely blocked the G1-induced increase in GLT-1 protein expression (Fig. 4B). The same inhibitors alone did not alter GLT-1 levels with the exception of AG1478 (EGFR inhibitor), indicating that the EGFR pathway is critical for GLT-1 regulation (Fig. 4B). The G1-induced increase in GLT-1 protein expression was completely abrogated by the Src inhibitor PP2 (Fig. 4C).

GPR30 Activation Induces Astrocytic GLT-1 Expression via the CREB Pathway—The GLT-1 promoter contains a cis-element for CREB binding (Fig. 5A). Accordingly, we investigated the role of the CREB pathway in the G1-induced GLT-1 expres-

![Figure 5](image-url)
GPR30 Regulation of GLT-1

**GPR30 Activation Regulates Astrocytic GLT-1 Expression via the NF-κB Pathway**—EAAT2 (GLT-1 human homolog) contains several NF-κB binding sites in its promoter region (Fig. 7A). Because EGF and neuronal factors regulate GLT-1 expression via the NF-κB pathway (32, 36), we investigated whether NF-κB signaling contributes to G1 regulation of GLT-1 expression. The NF-κB inhibitors, pyrrolidine dithiocarbamate (PDTC, 50 μM) or amino-4-(4-phenoxy-phenylethylamino)quinazoline (QNZ, 10 μM), significantly blocked the G1-induced increase in GLT-1 protein expression (Fig. 7B; p < 0.001). G1 significantly increased binding of both subunits of NF-κB, p50 and p65, to the GLT-1 promoter (p < 0.001) (Fig. 7C). G1 significantly increased NF-κB luciferase reporter activity (p < 0.05) and GLT-1 promoter activity in a time-dependent manner (Fig. 7D). Triple mutation of the NF-κB binding sites (−583/−272/−251) in the GLT-1 promoter significantly, although not completely, decreased the effect of G1 on the triple mutant GLT-1 promoter (p < 0.001), as well as basal reporter activity. Moreover, suppression of NF-κB activation by overexpression of IκBα blocked the G1-induced effect on NF-κB activation (Fig. 7D). These results indicate that NF-κB contributes to GPR30 regulation of GLT-1 expression.

**FIGURE 6.** G1 regulates GLT-1 expression and GLT-1 promoter activity via the CREB pathway. A, GLT-1 protein in astrocytes preincubated with 10 μM H89 or 1 μM KT5720 (PKA inhibitors) prior to G1 treatment for 24 h in the presence of H89 or KT5720; β-actin serves as a loading control. B, GLT-1 promoter activity as indicated by luciferase assay in astrocytes transfected for 16 h with GLT-1 luciferase reporter (EAAT2Pro-954) and treated for 1 h with H89 (10 μM) prior to G1 (2 μM) treatment for 24 h. C, GLT-1 promoter activity in astrocytes transfected with the GLT-1 promoter (EAAT2Pro-954) either alone or with dominant-negative CREB (A-CREB), followed by treatment for 20 h with dbcAMP (250 μM, a positive control) (left panel) or G1 (2 μM) (right panel). D, GLT-1 promoter activity as indicated by luciferase assay in astrocytes transfected with GLT-1 promoter lacking the CRE site (mutated on CRE site, 308m) and treated with G1 or dbcAMP for 20 h. The data are the means ± S.E. of three independent experiments. **, p < 0.01; ***, p < 0.001; ##, p < 0.01; ###, p < 0.001; NS, no significant difference; n = 4. ANOVA followed by Tukey’s post hoc test was used.

section. ChIP demonstrated that G1 enhances CREB binding to the GLT-1 promoter compared with CREB binding in untreated controls (Fig. 5B). dbcAMP was used as a positive control (Fig. 5B). As early as 5 min after its addition to the media, G1 induced CREB phosphorylation (Fig. 5C), which returned to levels indistinguishable from controls within 30 min. We confirmed that G1 increases CREB binding by luciferase assay using the CRE element from the GLT-1 promoter (p < 0.001; dbcAMP, positive control; Fig. 5D).

CREB is activated by the PKA pathway, which is activated by GPR30 (38), therefore we determined whether PKA contributes to this effect of G1. As shown in Fig. 6A, H89 (10 μM) or KT5720 (1 μM), specific PKA inhibitors, fully abrogated the G1-induced increase in astrocytic GLT-1 protein expression (p < 0.01; Fig. 6A). H89 also fully suppressed the G1-induced increase in GLT-1 promoter activity (p < 0.01; Fig. 6B). Suppressing the CREB pathway with dominant-negative CREB (A-CREB) abolished the G1-induced GLT-1 promoter activation (p < 0.001; Fig. 6C), matching the effect on dbcAMP-induced activation, indicating that CREB binding is entirely PKA-dependent (Fig. 6C). Furthermore, mutation in the CRE site of the GLT-1 promoter significantly decreased the basal GLT-1 promoter activity and abolished the G1-induced increase in GLT-1 promoter activity (p < 0.001; Fig. 6D), indicating a critical role for the CREB pathway in mediating G1-regulated GLT-1 expression.

**GPR30 Regulation of GLT-1**
GPR30 Activation Reverses Mn-induced Impairment in Astrocytic GLT-1 Expression and Glutamate Uptake—Exposure to excessive levels of Mn increases synaptic glutamate levels by decreasing the expression of astrocytic glutamate transporters (22, 39). Thus, treatment with manganese offers a unique experimental tool to determine whether a particular treatment that alters glutamate transporter function is neuroprotective. G1 (18 h of pretreatment) reversed the decrease in glutamate uptake induced by 6 h treatment with 250 μM Mn (p < 0.01) (Fig. 8A). Concomitantly, G1 also reversed the Mn-induced decrease in GLT-1 protein expression (Fig. 8B, Western blots; Fig. 8C, immunocytochemistry).

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GPR30 Activation Reverses Mn-induced Decrease in GLT-1 Promoter Activity via the NF-κB/CREB Pathway—Because TNF-α represses GLT-1 promoter activity via activation of the NF-κB pathway, we surmised that Mn might act through the same mechanism (40). Mn induced p65 nuclear translocation in a time-dependent manner (Fig. 9A), activated the NF-κB luciferase reporter 3 h after treatment (Fig. 9B), and repressed GLT-1 promoter activity (Fig. 9C). Because G1 acts through NF-κB and CREB to regulate GLT-1 expression, we investigated whether these relate to Mn-induced changes in expression. G1 blocked the Mn-induced nuclear translocation of NF-κB p65 (Fig. 9D) and reversed the Mn-induced decrease in GLT-1 promoter activity (Fig. 9E). Finally, G1 enhanced CRE luciferase reporter activity and reversed the Mn-induced reduction in CRE reporter activity (Fig. 9E).

DISCUSSION

Our findings offer novel insight into the molecular mechanism by which GPR30 regulates the expression and activity of astrocytic GLT-1. G1, a selective agonist of GPR30, enhanced GLT-1 protein and mRNA expression in a time- and concentration-dependent manner (Fig. 1), concomitant with increased astrocytic glutamate uptake (Fig. 1). GPR30 regulation of GLT-1 was confirmed by the findings that suppression of
GPR30 reduced GLT-1 and TGF-α mRNA levels and abrogated G1-induced GLT-1 expression (Fig. 2). The GPR30-induced increase in GLT-1 expression is mediated at the transcriptional level, via both NF-κB and CREB pathways (Figs. 5–7). Moreover, GPR30 activation reverses the Mn-induced impairment in astrocytic glutamate uptake and GLT-1 protein expression (Fig. 8). GPR30 also regulated GLAST expression in a similar fashion but to a lesser extent compared with GLT-1. The effects of G1/GPR30 appear to be specific to the astrocytic glutamate transporters, GLT-1 and GLAST, because it did not alter other brain proteins such as GAP43. The findings provide novel information on potential molecular/cellular targets for therapeutics to combat neurodegenerative diseases associated with excitatory neuronal injury.

E2 enhances glutamate uptake and GLT-1 expression in astrocytes (21, 35), yet there has been little progress in understanding the mechanism that mediates these effects. Our findings establish that E2-induced GLT-1 expression is predominantly mediated via GPR30, although there is also a contribution from the classical ERs, ER-α and ER-β (41). Consistently, we show that blocking G protein function fully abrogates the effect of G1 on GLT-1 protein expression but only partially reduces the effect of E2 (Fig. 3).

The role of GPR30 in mediating the effects of E2 in both physiological and pathological states has been investigated since it was cloned, approximately a decade ago (8). GPR30 mediates intracellular calcium release (42), regulates prolactin secretion (15), inhibits extracellular dopamine efflux (43), and attenuates serotonin signaling in the hypothalamus (44). G1 has also been shown to induce neuroprotection in several experimental models. For example, G1 exerts anti-inflammatory effects in experimental allergic encephalomyelitis (45), attenuates ischemia-induced hippocampal neuronal loss (15), and protects hippocampal neuronal cells from glutamate-induced neurotoxicity (46). At the other end of the spectrum, GPR30 activation has been suggested to promote tumorigenesis in a subset of cancers (12, 47).

Although GPR30 is known to regulate GLT-1 expression, the molecular mechanism of this effect at the transcriptional level has yet to be resolved. Given that GPR30 transactivates EGFR, which regulates GLT-1 expression through the NF-κB pathway (32, 40), NF-κB is a likely mediator. Our results indicate that the NF-κB pathway is intimately involved in the G1-induced enhancement of GLT-1 expression (Fig. 7). Both NF-κB p50 and NF-κB p65, which compose the predominant heterodimer form of NF-κB in the brain (48), appear to be involved in G1-induced GLT-1 regulation, supported by observations that both can induce GLT-1 expression (supplemental Fig. S2) and that the binding of both p50 and p65 to the GLT-1 promoter was enhanced by treatment with G1 (Fig. 7C).

G1 may activate NF-κB independently of its nuclear translocation while blocking Mn-induced p65 nuclear translocation (Fig. 9D). This G1 effect is distinctive, whereas E2 is also known to inhibit chemokine (CCL2) expression via NF-κB without blocking p65 nuclear translocation (49). On the other hand, ceftriaxone, which is known to stimulate GLT-1 mRNA and protein expression in several cellular and animal models (50, 51), has been reported to regulate GLT-1 expression via activa-
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FIGURE 10. Proposed mechanism of GPR30 activation of glutamate transporter function in astrocytes. GPR30 enhances GLT-1 expression via G protein and the cAMP/PKA pathway, transactivation of EGFR, and Src/MAPK/PI3K signaling from both GPR30 and EGFR. The transcription factors NF-κB and CREB mediate G1-induced GLT-1 expression in astrocytes.

In summary, the present study shows that GPR30 regulates GLT-1 expression by multiple pathways including 1) cAMP-PKA activation of CREB and 2) transactivation of the EGFR-PI3K-NF-κB pathway (Fig. 10). Our findings of G1-induced increased levels of GLT-1 mRNA and protein and glutamate uptake activity are consistent with the efficacy of GPR30 in mediating GLT-1 activity because silencing GPR30 suppressed GLT-1 expression as well as G1-induced increase of GLT-1 (Fig. 2). Accordingly, GPR30 offers a novel therapeutic target for modulating excessive synaptic glutamate levels, which are inherent to a plethora of neurodegenerative diseases.

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