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Brain Research 978 (2003) 213–222
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www.elsevier.com/locate/brainres

Research report

Glutamate-dependent glutamine, aspartate and serine release from rat cortical glial cell cultures

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Accepted 17 April 2003

Abstract

Glia play a pivotal role in glutaminergic excitatory neurotransmission in the central nervous system by regulating synaptic levels of glutamate and by providing glutamine as the sole precursor for the neurotransmitter pool glutamate to neurons through the glutamate–glutamine cycle. In the present investigation, we examined the influence of glutamate application on glutamine, serine and aspartate release from rat cortical glial cultures. The glial glutamate transporters rapidly cleared exogenously applied glutamate and this was accompanied by rapid increases in aspartate and glutamine, and a delayed increase in serine levels in the glial-conditioned medium. While glutamate-induced increases in glutamine and serine were sustained for up to 24 h, increases in aspartate lasted only for up to 6 h. The glutamate-induced increases in aspartate and glutamine were dependent both on the concentration and the duration of glutamate stimulus, but were largely insensitive to the inhibition of non-*N*-methyl-D-aspartate receptors or the metabotropic glutamate receptor 5. Inhibition of the glutamate transporter function by L-*trans*-pyrrolidine 2,4-dicarboxylate decreased the rate of glutamate uptake but not completely abrogated the uptake process, and this resulted in the attenuation of rate of glutamate induced glutamine synthesis. Dexamethasone treatment increased serine and glutamine levels in conditioned medium and increased glutamate induced glutamine release suggesting an upregulation of glutamine synthase activity. These results further substantiate coupling between glutamate and glutamine, and shed light on glutamate-dependent release of serine and aspartate, which may further contribute to excitatory neurotransmission.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Excitatory amino acids: pharmacology

Keywords: Glia; Glutamate–glutamine cycle

1. Introduction

The excitatory neurotransmission in the central nervous system involving glutamate (Glu) is a highly regulated process. The regulatory steps include: (1) exclusion by blood–brain barrier of the Glu in circulation, thereby rendering the brain an autonomous entity both for anabolism and catabolism of Glu [7,10], (2) juxtaposition of glutamatergic synapses with glial cells that are richly endowed with glutamate transporters which rapidly clear released Glu [5,15,33,36], (3) presynaptic localization of metabotropic glutamate receptor 2 (mGluR 2) which appears to regulate action-potential generated Glu release from neurons [35], (4) restricted localization of glutamine synthase (GS; [23]) and pyruvate carboxylase to astrocytes which are, respectively, involved in the conversion of Glu to glutamine (Gln) and de novo synthesis of Glu from glucose [9,11,12,19,20]. The metabolism of the major excitatory and inhibitory neurotransmitters in the brain, Glu and γ-aminobutyric acid (GABA), respectively, is intimately linked to a substrate cycle between neurons and astrocytes involving Gln [2,17,25,28,30,37]. The importance of glutamine cycle in vivo is demonstrated by the recent in vivo ^{13}C and ^{15}N NMR studies in rodents and ^{13}C NMR in humans which indicate that glutamine synthesis is substantial and that the total glutamate–GABA–glutamine cycling flux, necessary to replenish neurotransmitter Glu and GABA represents nearly 80% of the net Gln synthesis [3,4,25]. Given the central role of astrocytes in these processes, the conventional view of neurotrans-

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0006-8993/03/$ – see front matter
doi:10.1016/S0006-8993(03)02841-5

mission via bi-partite neuronal synapses has been challenged to include tripartite synapses involving neuron–astrocyte–neuron interactions (reviewed in Refs. [16,24]).

A number of studies, using radioactive substrates, have examined the inter-relationship between Glu uptake and intracellular Gln synthesis using either radiolabeled Glu or glucose [9,22]. From these studies, it appears that extracellular concentrations of Glu influence the metabolic fate of Glu [20]; for example, at extracellular concentrations of ≤0.1 mM, 83% of Glu taken up by astrocytes is metabolized to Gln, where as at a higher concentration of 0.5 mM, only 43% of Glu is converted into Gln [21]. In addition, Glu can also be converted to aspartate (Asp) via the tricarboxylic acid (TCA) cycle [21,36]. Since Gln release from astrocytes is an essential part of Glu–Gln cycle, release of Gln into the extracellular space is the most direct method to assess the inter-relationships between Glu uptake and Gln biosynthesis. Therefore, in the present investigation, we have conducted detailed investigation on dose response and time course relationships between Glu uptake into, and release of Gln and Asp from, rat cortical glia. In addition, we also examined the effects of inhibition of Glu uptake by L-*trans*-pyrrolidine-2,4-dicarboxylic acid (PDC), a putative Glu transport inhibitor [8,41], on Glu–Gln cycle, and effects of dexamethasone, a known inducer of GS activity [14] on Glu-induced Gln release.

2. Materials and methods

2.1. Astrocyte cultures

Mixed rat cortical glial cells were prepared from neonatal Sprague–Dawley rat pups (0–2 days) as per methods described earlier [43]. Rat brain cortices were isolated under sterile conditions, minced and gently triturated using fire-polished Pasteur pipettes. Cells were plated at a density of 500 000 cells/well in 24-well plates precoated with poly-L-lysine and grown incubated at 37 °C in an atmosphere with 5% CO_2 at 95% humidity in the presence of growth medium containing DMEM (Gibco, USA) with 25 mM glucose and 2 mM L-glutamine supplemented with 10% heat-inactivated FBS (Omega Scientific, CA). The culture medium was changed 6 days later and twice-a-week thereafter. Immunohistochemical analysis indicated that greater than 85% of the cells were glial fibrillary acid protein positive with smaller amounts of microglia and oligodendrocytes.

All chemicals of highest purity were purchased from Sigma (St. Louis, MO, USA), Fisher Scientific (USA) or from Tocris Cookson (Ballwin, USA).

2.2. Analysis of Glu, Gln, Asp and Serine (Ser) in supernatants

All biochemical experiments were conducted on 15–20-day-old cultures with the day of dissection considered as day 1. Prior to the application of pharmacological treatments, cultures were extensively washed to remove tissue culture media (three to four times with 1 ml warm Earle's balanced salt solution (EBSS containing 5 mM glucose; Gibco)/well of 24-well plate, followed by the addition of 1 ml of warm EBSS/well of 24-well plate and incubation for 30 min at 37 °C. After the removal of the medium, pharmacological treatments were applied in 0.5 ml EBSS/well. At the end of selected periods of exposure, supernatants (glial conditioned media; GCM) were collected, filtered through 0.2-μm filters (AllTech, Deerfield, USA), and aliquots were placed in vials for amino acid analysis by high-pressure liquid chromatography (HPLC)-fluorescence detection following pre-column derivatization with *o*-phthalaldehyde (OPA). The cells were digested in 0.5 ml of 0.5 N NaOH and protein levels were determined by bicinchoninic acid kit (Pierce, Rockford, USA). Levels of amino acids were quantified by external standard methods using synthetic L-amino acid standards from Sigma.

The HPLC system consisted of SCL 10A VP system controller, two LC-10 Ad VP pumps, DGU-14 solvent degasser, SIL 10-ADVP autoinjector with Pelletier cooling, RF-10 AXL fluorescence detector, CTO-10 AVP column heater (set at 40 °C; all from Shimadzu, Gaithersburg, USA). The mobile phase consisting of varying proportions mobile phase A (phosphate buffer (100 mM, pH 6.40) containing 20% methanol) and mobile phase B (90% HPLC-grade methanol in HPLC-grade water) was pumped through a Hypersil Elite C18 column (150×4 mm, 5-μm particle size) preceded by a Security Guard® with C18 frit (5 μM particle size; both from Phenominex, Fullerton, CA) at a flow rate of 1 ml/min using a binary gradient program. The binary gradient mixing program consisted of the following steps: % B: 7.5% from 0 to 11 min, 20% B by 12 min, 40% by 17 min and maintained until 19 min, reduce to 7.5% by 20 min and maintain in this condition until the next injection. The pre-column derivatization was initiated by the addition of 10 μl of OPA solution (27 mg of OPA dissolved in 1 ml methanol containing 10 μl of 2-mercaptoethanol and diluted to 10 ml with the addition of 9 ml of 0.1 M sodium tetraborate buffer) to 25-μl aliquots of GCM. After 2 min of reaction, a 20-μl sample was injected onto the HPLC column by a programmable autoinjector (Shimadzu).

2.3. Lactate dehydrogenase (LDH) measurement

Levels of LDH, a marker for cellular injury, were measured by using commercially available Cytotoxicity Detection Kit (Roche Diagnostics). This assay measures LDH-mediated conversion of tetrazolium salt to a red formazan product.

2.4. [^{3}H]D-Serine release

Mixed glial cultures were exposed to [^{3}H]D-serine (26.8 Ci/mmol, NEN; 100 nM; for 60 min) in EBSS and

repeatedly washed with warm EBSS. Pharmacological agents were applied at appropriate concentrations for a period of 30 min in EBSS (0.5 ml/well). Supernatants were collected and lysed with 0.5 mL of 0.1% Triton X100 in EBSS. Radioactivity in supernatants and cell lysates was determined by liquid scintillation spectrometry. Radioactivity in supernatants was normalized to total radioactivity (i.e., lysates+supernatants) to provide fractional efflux.

2.5. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by Neuman–Keuls' post-hoc analysis.

3. Results

Levels of Glu, Asp, Ser and Gln released into GCM were normalized to protein content in each well and expressed as pmol/ml/μg of protein. The concentration of Glu, exposed to glia, is expressed as μM.

3.1. Asp, Ser, Glu and Gln in glial conditioned medium (GCM) following exposure to Glu (300 μM)

3.1.1. Asp

The concentrations of Asp in 24 h supernatants were in the low pmol/ml range (<5 pmol/ml per μg protein) and this may reflect either a limited basal release of Asp from glia into GCM and/or rapid uptake of released Asp from GCM by glia resulting in lower steady state levels in the extracellular milieu. Asp levels rapidly increased in GCM after Glu application with significant increases seen as early as 15 min and peak release was observed at 1 h. Asp levels in GCM decreased rapidly thereafter reaching pre-Glu application levels by 6 h and remained at these levels over a 24-h period (Fig. 1A)

3.1.2. Ser

The HPLC methodology employed here does not discriminate between L- and D-serine, therefore the levels reflect that of a racemic mixture, hence referred to as Ser. Glia contain D-Ser and are known to release it into GCM

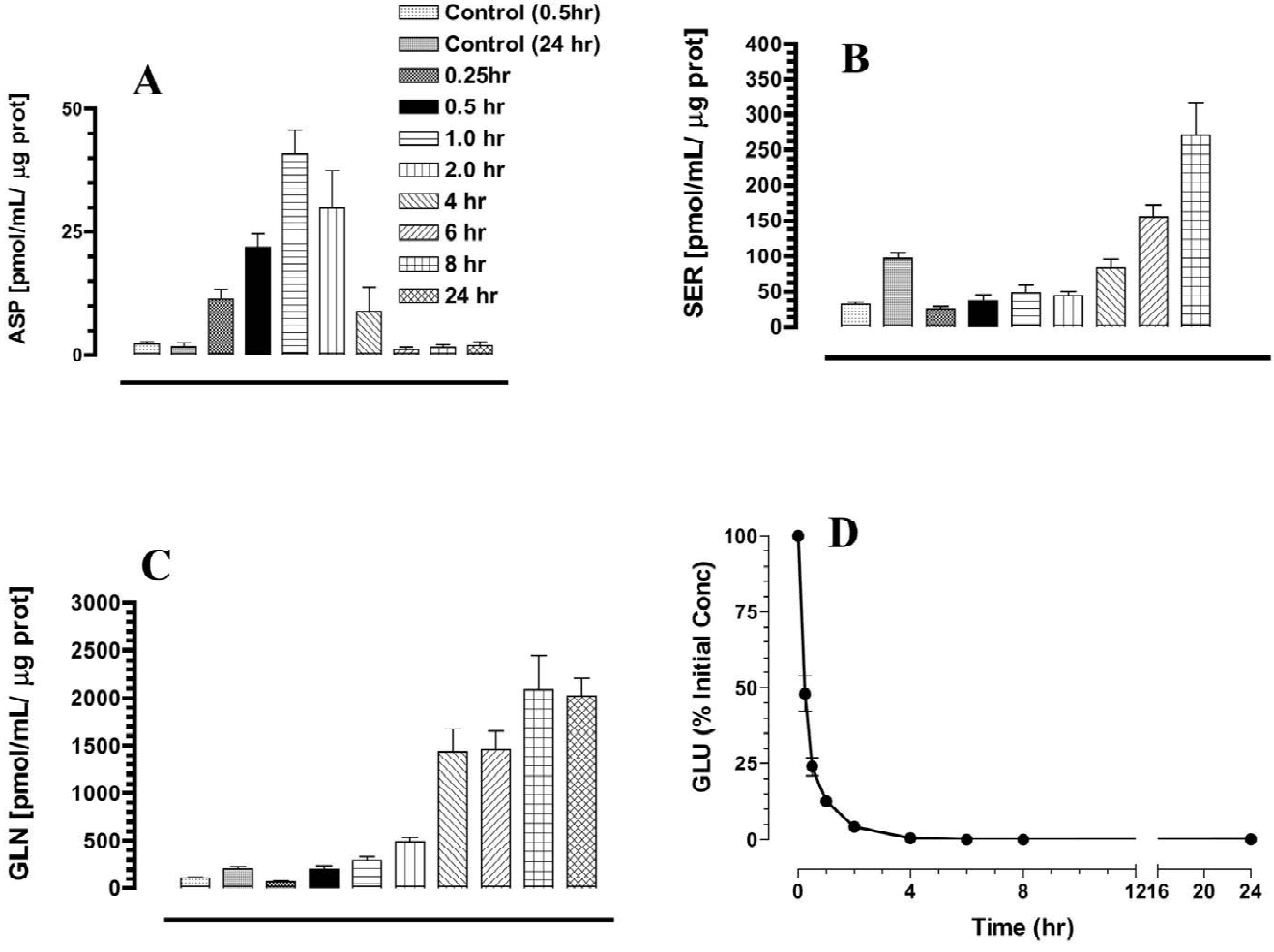

Fig. 1. Time course of release of aspartate (Asp), serine (Ser) and glutamine (Gln) into glial-conditioned medium following the addition of glutamate (Glu, 300 μM). The levels of amino acids in the glial conditioned medium from glia cultured in 24-well plates were quantitated by HPLC methods, represented as pmol/ml, normalized to protein content in μg. Values represent mean+S.E.M., n=9–24 replicates.

[31,39]. Extracellular levels of D-Ser are nearly 30% of the L-Ser levels [42]. In addition, glia are known to take up glycine and release Ser through the Gly–Ser cycle [39]. The concentrations of Ser in 24 h GCM were approximately 100 pmol/ml per μg protein and glial cells appeared to release Ser quite avidly as evidenced by an increase of approximately 30 pmol/ml per μg protein within 30 min of application of fresh EBSS to glia. Astroglia are known to release significant amounts of Ser with an initial rate approaching 600 pmol/min per mg of protein [38,39]. In separate experiments, basal fractional efflux of [^{3}H]D-serine from pre-labeled glia was found to be in the range of 5–10% of the total intracellular radioactivity (data not shown), further supporting a higher basal D-Ser efflux from glia. Glu application increased Ser release into the GCM with a delayed onset; measurable increases were seen at 4 h and Ser levels continued to increase to approximately 250 pmol/ml per μg protein by 24 h. These results are consistent with Glu induced D-Ser release from astrocytes [34]. The delayed onset of Glu-induced Ser release is consistent with it being a consequence of Glu-induced metabolic effects rather than a consequence of Glu receptor activation (Fig. 1B).

3.1.3. Gln

Gln levels in 24 h GCM were approximately 200 pmol/ml per μg protein. Following Glu application, Gln levels in GCM increased rapidly and by 4 h reached approximately 1500 pmol/ml per μg protein. Peak levels of approximately 2000 pmol/ml per μg protein were reached at 8 h and were maintained over a 24-h period after Glu exposure, thereby representing nearly a 10-fold increase in Gln release in to GCM (Fig. 1C).

3.1.4. Glu

Twenty-four hour GCM contained extremely low levels of Glu (0.5–2 pmol/ml per μg protein). This contrasts with much higher levels of Ser and Gln levels in the GCM. These results suggest minimal Glu release from glia under basal conditions and are consistent with low levels of Glu release from astrocytes [19]. Following the application of Glu, glia rapidly cleared extracellular Glu. Kinetic analysis indicated that of Glu clearance by Glia followed a first order kinetics with a half life of Glu in the range of 25–35 min with a kinetic constant of clearance of 231 pmol/mg protein per min (Fig. 1D).

3.2. Concentration-related effects of Glu exposure to glia on Asp, Ser and Gln release into GCM and Glu uptake

Concentration-related effects of Glu (30–3000 μM) exposure to glia on Asp, Ser and Gln release into GCM were examined both after 1 (Fig. 2A) and 24-h exposure (Fig. 2B). In addition, the levels of Glu remaining in the GCM were also measured at both time points (Fig. 2C).

3.2.1. Asp

Glu exposure induced Asp release into the GCM both at 1 and 24 h (Fig. 2A) and this increase was related to the concentration of Glu stimulus. Interestingly, Glu (1000 and 3000 μM) exposure sustained Asp release that lasted for up to 24 h and these results contrast with a relatively transient increases seen at lower concentrations (Figs. 2A and 1A). These results suggest that higher concentrations can maintain Glu entry into TCA cycle leading to sustained biosynthesis and release of Asp into extracellular milieu.

3.2.2. Ser

One hour exposure of glial cultures to Glu increased Ser release into in the GCM only at the highest concentration of Glu, i.e., 3000 μM, with no effect at lower concentrations. A 24-h exposure to Glu modestly increased Ser release into in the GCM, and this pattern of increase was related to the concentration of Glu applied to glial cultures. At the highest concentration of Glu (3000 μM), 24 h GCM contained nearly 600 pmol/ml per μg protein of Ser. Once again, the delayed time course of Glu-induced Ser release into GCM suggests a metabolic effect of Glu rather than a receptor-mediated effect. Consistent with this hypothesis, Glu-induced Ser release was not sensitive to inhibition of non-NMDA receptors by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 100 μM) or inhibition of metabotropic glutamate receptor 5 (mGluR5) by 2-methyl-6-(phenylethynyl)pyridine (MPEP; 10 μM; data not shown).

3.2.3. Gln

Exposure of glia to varying concentrations of Glu for 1 or 24 h evoked Gln release into GCM in a concentration-related manner. At either time period of Glu exposure over a wide range of concentrations (30–3000 μM), there was no clear indication of saturation of release of Gln into GCM.

3.2.4. Glu

GCM from 24-h exposure of glia to Glu (30–1000 μM) contained less than 3 μM of Glu. However, when the concentration of Glu was increased to 3 mM, the clearance of Glu by glia appeared to be impaired resulting in higher levels of Glu in the conditioned medium. Nevertheless, these results, in toto, attest to the marked avidity of Glu uptake process by the glial Glu transporters.

3.2.5. Investigation of Glu-induced toxicity to glial cultures

Levels of lactate dehydrogenase (LDH) in GCM samples were quantitated to measure Glu-induced damage to glia (data not shown). Exposure of glia to Glu did not

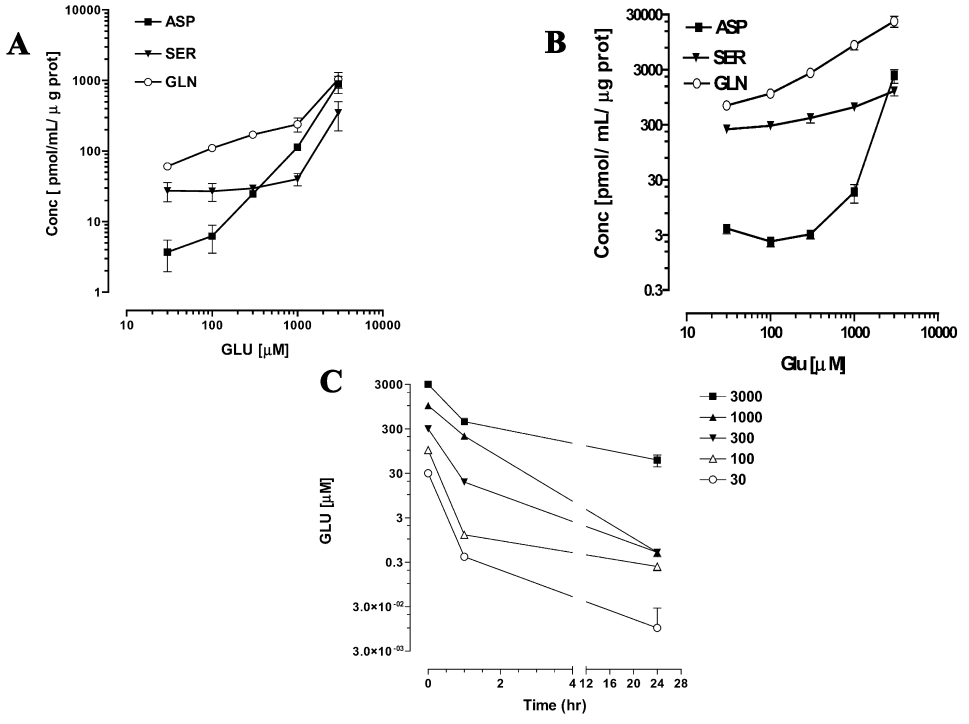

Fig. 2. Concentration related effects of Glu application on the release of Asp, Ser and Gln into glial-conditioned medium and the clearance of exogenously applied Glu: (A) 1 h after Glu application, and (B) 24 h after Glu application. Values represent mean+S.E.M., $n=12$ replicates. Levels of Gln, Ser, Asp and Glu in glial conditioned medium derived from control condition at 60 min were (pmol/ml per µg of protein; Mean±S.E.M., $n=12$): 70±9.5, 67.3±9.2, 1.9±0.43 and 0.56±0.07, respectively. The corresponding values from glial conditioned medium derived from 24 h control condition were (pmol/ml per µg of protein; Mean±S.E.M., $n=16$): 571±71, 270±33, 3.8±0.8 and 1±0.18, respectively.

increase LDH levels in GCM over those seen with exposure to EBSS and these results were corroborated by trypan blue exclusion or visual examination of health of the cultures. However, exposure of glial cultures to Glu (30 µM) in the presence of cyclothiazide (25 µM), a benzothiadiazide that prevents rapid desensitization of AMPA receptors, induced highly significant LDH efflux that is accompanied by increased trypan blue staining of injured glia. Inclusion of CNQX (30 µM), an AMPA antagonist, during the exposure to Glu (30 µM) and cyclothiazide (25 µM), completely prevented LDH efflux and trypan blue staining. Taken together, these results argue that changes in amino acids in GCM in response to Glu exposure do not reflect cellular injury.

3.3. Effect of inhibition of Glu transporter function on Glu induced Gln release into GCM

Since Glu application increased Gln release from glial cultures following uptake and subsequent conversion into Gln, we investigated the effect of inhibition of Glu transport function by L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC; 3 mM; [8,41]) on this process (Fig. 3A–D). We reasoned that PDC, by inhibiting Glu uptake, will markedly attenuate Glu-induced Gln release and that such a result will establish the tight functional coupling between Glu uptake and Gln release. In order to kinetically characterize the process, glial cultures were exposed to PDC or vehicle for 30 min, and exposed to fresh EBSS containing Glu (300 µM) in the presence or absence of PDC (3 mM) for 1, 4 or 24 h. PDC application itself significantly increased Glu levels in GCM from approximately <1 to 5–8 pmol/ml per µg protein over the time period of 1–24 h (Fig. 3A). Since these increases occurred in the absence of any exogenous Glu, such increases are likely to reflect actions of PDC through heteroexchange via the transporter leading to release of Glu from glia. In the absence of PDC, glial cells rapidly cleared Glu from an initial concentration of 300 µM to approximately 300 nM, i.e., 1000-fold clearance, by 24 h. In the presence of PDC, the rate of decline of exogenously applied Glu markedly diminished. At all three time points examined, i.e., 1, 4 and 24 h, PDC treatment resulted in 3–15-fold higher levels of Glu in the GCM as compared to the Glu alone condition. However, at a concentration as high as 3 mM, PDC treatment did not completely abolish Glu uptake by glia. This is evidenced by residual concentrations of approximately 15 µM of Glu in 24 h GCM upon exposure to PDC (3 mM) and Glu (300 µM). These data suggest that PDC at the concentration of 3 mM did not provide a complete inhibition of Glu uptake over a 24-h period. Since PDC itself is a substrate for Glu transporter via heteroexchange [41], the effective concentrations of PDC are likely to decrease over time upon exposure to glial cultures resulting in a time-dependent

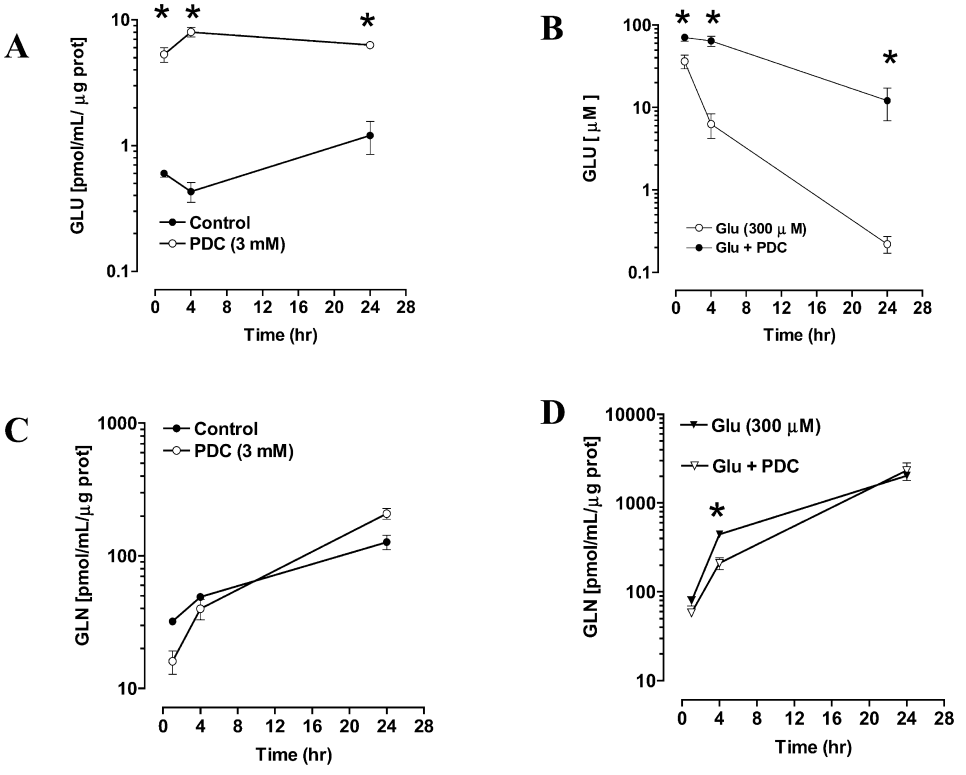

Fig. 3. Effect of inhibition of Glu transporter, L-*trans*-pyrrolidine-2,4-dicarboxylate (PDC) on Glu clearance and Gln release into glial-conditioned medium. Glia were pre-treated with PDC (3 mM) or vehicle 30 min prior to the removal of conditioned medium and application of fresh EBSS containing Glu (300 μM) alone or in combination with PDC (3 mM). PDC treatment significantly attenuated the rate of clearance of Glu by glia (A) and only affected the release of Gln into glial conditioned medium at 4 h, but not at 24 h. *$P<0.05$ versus no PDC treatment condition. Values represent (mean±S.E.M., $n=6–12$ replicates.

loss of inhibition of Glu transport. PDC treatment tended to decrease basal Gln release at 1 h and increase them at 24 h (Fig. 3C). Similarly, PDC attenuated Glu-induced Gln release only at 4 h, but not at 24 h (Fig. 3D).

3.4. Dexamethasone regulation of glutamine release into GCM

The GS activity is known to be up-regulated by number of pharmacological agents, including the glucocorticoid, dexamethasone (Dex; [14]). Since increased GS activity should reflect in increased extracellular release of Gln into GCM, we examined the influence of Dex on Gln release. Twenty-four hours of exposure to Dex (1 μM) resulted in a 2-fold increase in Gln levels in the GCM (Fig. 4A). Surprisingly, these increases in Gln were also accompanied by a 2-fold increase in Ser levels into GCM (Fig. 4B). These results are consistent with similar observations from Verleysdonk and Hamprecht [38] who reported Dex-induced increases in Ser release into GCM, albeit after 80 h of Dex treatment. There were no discernible changes in Asp and Glu levels in GCM (data not shown). As a direct test for Dex-induction of GS activity, Gln levels in GCM were measured after exposing glial cultures to Glu (300 μM) with or without an overnight priming with Dex (1 μM). As expected, GCM from Dex-treated glial cultures contained significantly higher levels of Gln as compared to those derived from naïve glia, further supporting glucocorticoid regulation of GS activity (Fig. 4C).

Regulation of Asp, Ser and Gln release by Glu from glia is summarized in Fig. 5.

4. Discussion

The Glu–Gln cycle is considered the major pathway for Glu recycling in the mammalian brain and in vivo NMR studies document that the brain energy demands are tightly coupled to the recycling of neurotransmitter Glu via Gln [3]. In order to support neuronal energy demands, Gln synthesized in glia must be released into the extracellular milieu for subsequent uptake by neurons. In the present investigation, we examined the influence of Glu on release of Ser, Asp and Gln from glia. These results suggest a highly regulated interaction between the availability of Glu for uptake by glia and release of Ser, Asp and Gln from glia in to the extracellular space. The temporal profiles of changes of these three amino acids differed quite dramatically with Asp showing rapid and short-lasting increases, followed by sustained elevations both in Gln and Ser.

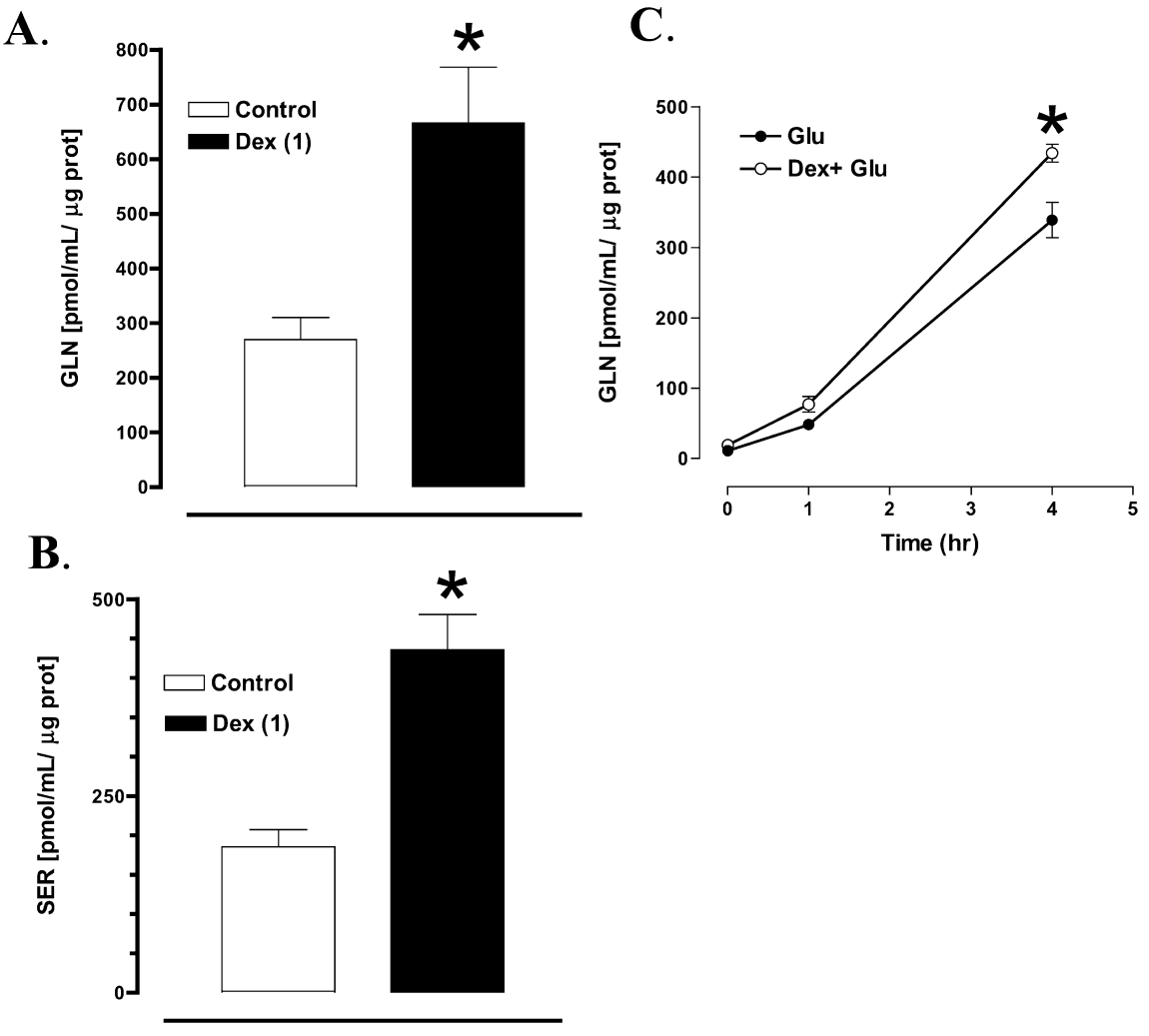

Fig. 4. Dexamethasone (1 μM) pretreatment increases Gln and Ser release into glial-conditioned medium and increases conversion of Glu to Gln. Following 24 h incubation of glial cells with Dex, conditioned medium was removed for Gln and Ser analysis (A,B) and glia were exposed to fresh EBSS with Dex and Glu (300 μM) and Gln release into the medium at 1 and 4 h after Glu application were measured (C). Values represent, mean+S.E.M., n=6–12 replicates. *P<0.05 versus control condition.

We have used primary glial cell cultures as the model system in which astrocytes constituting the majority with other glial populations such as microglia and oligodendrocytes constituting the minority. While we cannot rule out the contribution of microglia to the biochemical effects under investigation, differences between microglia and astrocytes: (1) relative absence of high-affinity Glu uptake in microglia [26], and (2) exclusive presence of glutamine synthase in astrocytes but not in microglia [23], suggest that astrocytes are the likely contributors to the observed effects. In addition, mouse microglia release more Glu than rat microglia and the magnitude of Glu release was entirely dependent on the amount of Gln exposure [26].

The carbon skeleton of extracellular glutamate is largely used for conversion into lactate and aspartate [38,39] and this is accomplished by the entry of Glu into the TCA cycle via 2-oxoglutarate. This pathway generates maleate and oxaloacetate, both of which can be converted into pyruvate via mitochondrial and cytosolic enzymatic machinery. Additionally, oxaloacetate can be transaminated to Asp by aspartate aminotransferase. Addition of Glu (500 μM) to glial cultures has been shown to increase intracellular Asp levels and this effect of Glu was potentiated by the co-application of Gln, which itself did not affect glial Asp synthesis and release [39]. The Glu-induced increases in Asp levels into GCM observed in the present investigation are consistent with the known entry of Glu into TCA.

Consistent with literature reports [38], GCM contained significant amounts of Ser implying release of Ser. Release of Ser from glia into extracellular milieu comes from a variety of sources: (1) biosynthesis from glucose, (2) via the glycine–serine cycle [38,39], and (3) proteolytic degradation. The biosynthesis of Ser from glucose proceeds via the phosphorylated pathway involving three key enzymes, (1) 3-phosphoglycerate dehydrogenase

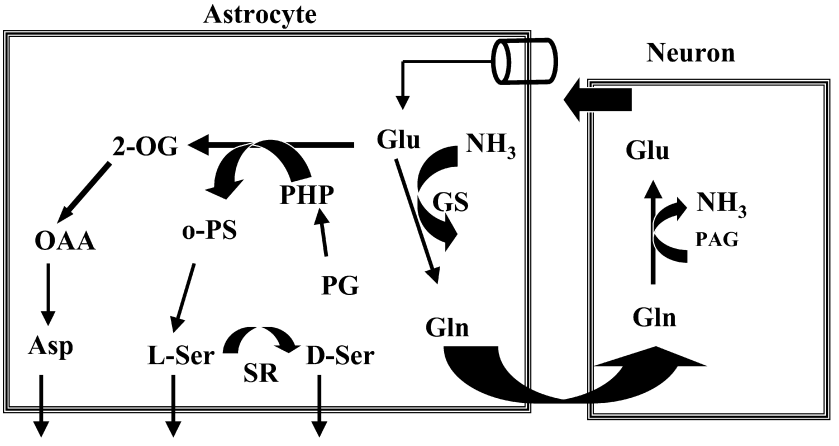

Fig. 5. Schematic model to encompass the effects of Glu on Asp, Ser and Gln release into astrocyte conditioned medium. Glu taken up by glial transporters converted into Gln by the action of glutamine synthase (GS) for release into the glial-conditioned medium. Neurons take up Gln and convert it into Glu via the actions of phosphate activated glutaminase (PAG). In glia, Glu also enters into the tricarboxylic acid cycle (TCA) to sequentially form 2-oxaloglutatare (2-OG) and oxaloacetic acid (OAA) and finally to Asp. In addition, Glu can also serve as an amino group donor in the phosphorylated pathway of Ser biosynthesis. 3-D-Phosphoglycerate (PG) undergoes dehydrogenation to phosphohydroxypyruvate (PHP) which acts as substrate for phosphoserine aminotransferase to form *o*-phosphoserine (*o*-PS). Phosphoserine undergoes dephosphorylation via phosphoserine phosphatase to generate L-Ser. Glia contain serine racemase which converts L-Ser to D-Ser, both of which are released into glial-conditioned medium.

which converts 3-D-phosphoglycerate to phosphohydroxypyruvate (PHP), (2) phosphoserine aminotransferase which converts PHP to *o*-phosphoserine (*o*-PS), and (3) phosphoserine phosphatase, which dephosphorylates *o*-PS to Ser (See Fig. 5; [41,45]). Phosphoserine aminotransferase catalyzed conversion of *o*-PS to Ser requires an amino acid to serve as a donor for the amino group. Glu is an effective amino group donor and others include alanine, aspartate and leucine [38]. Therefore, in the presence of glucose, Glu can effectively support Ser synthesis. In addition, glycine can also serve as a precursor for Ser; however, since the experiments were conducted in EBSS devoid of any exogenous glycine, the only source of amino group is derived from the exogenously added Glu. While it is known that protein degradation can increase Ser release from glial cultures [38], in the presence of glucose, protein degradation is likely to be a minor pathway. We observed that the glucocorticoid dexamethasone increased Ser release in to GCM and this effect may be related to its abilities to mobilize intracellular nitrogen sources for the provision of the serine amino group [18].

Glia are unique in that they take up Ser (both L- and D-forms) and also contain serine racemase, a key enzyme that can racemize L-Ser into D-Ser [31,34,39], and release both isomers. Biochemical and electrophysiological studies demonstrate an obligatory co-agonist and neuromodulatory role for D-serine in the excitatory neurotransmission through the *N*-methyl-D-aspartate receptors (NMDAR; [1,44,45]). Results from the present investigation demonstrate that Glu-regulates Ser release into extracellular milieu, thereby providing another potential avenue for regulating neurotransmission via the NMDAR. Glu acting via non-NMDAR is known to evoke [^{3}H]D-serine release [31]. Glial cultures express two classes of functional glutamate receptors: (1) metabotropic Glu Receptors, (mGluR5; [27]) and CNQX-sensitive ionotropic (non-NMDA) glutamate receptors [6]). The lack of effect of selective antagonists of these two classes of glutamate receptors on Glu induced Ser release into GCM, coupled with a delayed onset of Glu-induced Ser release support the idea that the observed effects are more likely to reflect metabolic rather than receptor-mediated effects of Glu.

In an attempt to elucidate a relationship between Glu uptake and release of Gln, Ser and Asp, we have employed PDC, a well known inhibitor of transport of Glu via the known Glu transporters (EAAT1-5; [8]). We reasoned that PDC, by inhibiting Glu uptake, would significantly attenuate Glu induced Gln biosynthesis and release. Although PDC slowed the clearance of exogenously applied Glu from GCM, it did not prevent the uptake process completely. In the absence of sustained, complete inhibition of uptake process, glia appear to clear Glu and release Gln into GCM. The relative lack of effect of PDC on Glu induced Gln release was surprising. However, the mechanism of action PDC does provide some clues. PDC treatment to naïve glia increased Glu levels in the GCM and these results can only be explained through release of Glu from glia mediated by a heteroexchange of PDC for Glu through the glial Glu transporter. By the use of glutamate transporters functionally reconstituted in liposomes, Volterra et al. [41] showed directly that PDC activates carrier-mediated release of glutamate via heteroexchange. This would predict that the inhibition of Glu by PDC will decrease in a time-dependent manner and that glia will clear most, if not all of the exogenously applied Glu. In addition, the time-dependent loss of inhibition of Glu transporter function would result over all Gln release in PDC treated glia that is some what similar

to that seen from naïve glia. Consistent with this observation, PDC attenuated Glu-induced Gln synthesis at earlier time points (4-h post Glu application) but not at later time points (24-h post Glu application). Nevertheless, the dose- and time-related effects of Glu on Gln release support the tight coupling between Glu uptake and Gln biosynthesis and release. The profile of Gln release seen in the present investigation supports similar findings using radiolabeled Glu [22].

Glutamine synthase (GS) is exclusively localized in glia and is subject to regulation by a variety of factors, including corticosteroids, adrenergic agonists, cyclic AMP, epidermal growth factor, insulin, thyroid hormones and by cytokines [13]. As such, regulation of GS activity is likely to show regional brain differences. GS also plays a key role in ammonia detoxification in the central nervous system by using it to synthesize Gln from Glu. It is believed that regulation of GS activity helps in normal Glu homeostasis in the nervous system. Impairments in Glu–Gln cycle have been linked to alternations in mood and behavior, disturbances in visual function, sleep patterns and amnesia. GS enzyme is extremely sensitive to oxidative damage and conditions such as Alzheimer's disease [11]. Interestingly, an aberrant expression of GS in neuronal population in AD has been documented [32], indicating profound changes in the plasticity of this key enzyme. Glucocorticoids, in addition to their effects on GS activity, are also known affect Glu transporter function in a region-selective manner [29,40]. The induction of GS enzyme by glucocorticoids may, therefore, represent a regulatory mechanism in response to cellular stress and injury.

The glia are the predominant cell types in the central nervous system. For a long time, these cells were thought to be non-excitable and therefore exclusively relegated to the task of providing nutritional and structural support to neurons. This view also supported the assumption that neurons alone contribute to synaptic transmission. An increasing understanding of the role of glia suggests that communication between glia and neurons is bi-directional (see commentary by LoTurco [16]). In addition, glia are now known to be endowed with a variety of ion channels, G-protein coupled receptors for various neurotransmitters and peptides, and transporters to terminate synaptic actions of neuronally released transmitters [16]. Glia exhibit an unique form of neuronally driven excitability [24] resulting in calcium oscillations, Glu release, which then signals to neighboring neurons, thereby modulating neurotransmission. The existence of Glu-regulated release of glial Ser, an obligatory co-agonist for the *N*-methyl-D-aspartate receptors, further underscores the critical role of glia in excitatory neurotransmission [44]. In addition, the functional coupling between Glu–Gln cycle along with Glu-induced Asp and Ser release from glia suggest the existence of an anaplerotic pathway in which glia play a central initiator role. Given the pleiotropic roles glia play in the nervous system, the conventional view of bi-partite neuronal synapses must include a tri-partite synapse involving neuron–astrocyte–neuronal synapses. The role of glia in the regulation of synaptic transmission is likely to exhibit regional differences in the mammalian nervous system, in part influenced by the region-specific glutamatergic innervation.

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