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Mechanisms of GABA Release from Human Astrocytes

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KEY WORDS
GABA transporters; glutamate; glutamate receptors; glycine; D-serine; GABA receptors; calcium chelation

ABSTRACT
We have previously demonstrated that human astrocytes are GABAergic cells. Throughout the adult human brain, they express the GABA synthesizing enzyme GAD 67, the GABA metabolizing enzyme GABA-T, and the $GABA_A$ and $GABA_B$ receptors. GABA modulates the actions of microglia, indicating an important role for astrocytes beyond that of influencing neurotransmitter function. Here we report on the mechanisms by which astrocytes release GABA. Astrocytes were found to express the mRNA and protein for multiple GABA transporters, and multiple receptors for glutamate, GABA, and glycine. In culture, untreated human astrocytes maintained an intracellular GABA level of 2.32 mM. They exported GABA into the culture medium so that an intracellular-extracellular gradient of 3.64 fold was reached. Inhibitors of the GABA transporters GAT1, GAT2, and GAT3, significantly reduced this export in a Ca^{2+}-independent fashion. Intracellular GABA levels were enhanced by treatment with the GABA-T inhibitors gabaculine or vigabatrin. Treatment with glutamate increased GABA release in a concentration-dependent fashion. This was partially inhibited by blockers of *N*-methyl-D-aspartate and kainate receptors. Conversely, glycine and D-serine, co-agonists of NMDA receptors, enhanced the GABA release. GABA release was accompanied by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and was reduced by adding the Ca^{2+} chelator, BAPTA-AM to the medium. These data indicate that astrocytes continuously synthesize GABA and that there are multiple mechanisms which can mediate its release. Each of these may play a role in the physiological functioning of astrocytes.

INTRODUCTION

Recently we reported that astrocytes, throughout the adult human brain, express the GABA synthesizing enzyme glutamic acid decarboxylase (GAD) 67, the GABA metabolizing enzyme GABA-transaminase (GABA-T), and the $GABA_A$ and $GABA_B$ receptors. We further reported that cultured human astrocytes strongly express the mRNAs and proteins for these molecules indicating that they possess the properties of GABAergic cells. Cultured microglia in contrast do not express GAD, but they do express GABA-T and the $GABA_A$ and $GABA_B$ receptors, indicating that they possess GABAceptive but not GABAergic properties. We demonstrated that GABA suppresses the reactive response of both astrocytes and microglia to inflammatory stimulants and that this reduces the toxicity of their conditioned medium to human neuroblastoma cells. These results indicate an important role for GABA in modulating glial functions (Lee et al., 2011).

In this report we explore the mechanisms by which cultured human astrocytes produce and release GABA. We show that they continuously synthesize GABA, maintaining a surprisingly high intracellular level. They export excess GABA into the surrounding media. The release is enhanced by adding to the media glutamate, glutamate receptor stimulants, or GABA receptor stimulants. Such stimulant-dependent release involves enhancement of intracellular calcium levels and is inhibited by calcium chelation.

MATERIALS AND METHODS

Materials

All reagents were purchased from Sigma (St. Louis, MO) unless stated otherwise. BAPTA-AM was purchased from Anaspec (Fremont, CA). The 2-aminoethoxydiphenyl borate (2-APB), NNC 711, NNC 05-2090, and (S)SNAP-5114 were purchased from Tocris Biosciences (Ellisville, MO). Diazepam was a gift from Dr. Timothy Murphy (The University of British Columbia, Vancouver, Canada).

Cell Culture and Experimental Protocols

Human astroglial and microglial cells were isolated from surgically resected temporal lobe tissue. Protocols were performed as described previously in detail (Lee et al., 2009). Briefly, tissues were dissected and incubated in 0.25% trypsin solution. They were pelleted, resuspended in medium, and passed through a 100 μm nylon cell strainer. They were resuspended in DMEM/F12 medium and plated onto tissue culture plates to achieve adherence of microglial cells. The non-adherent astrocytes along with myelin debris were transferred into other culture plates. Adherent astrocytes were allowed to grow by replacing the medium once a week. Human

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TABLE 1. Primer Sequences and Amplicon Sizes of Subunits of NMDAR, AMPAR, KAR, mGluR1, GlyR, $GABA_AR$ (α1 and γ1), and PBR

Genes	Primer sequences	Amplicon size (bp)	References
NR1	Forward 5′-AGGAACCCCTCGGACAAGTT-3′ Reverse 5′-CTCTCCAGTCGTCACCAGGT-3′	237	Stepulak et al. (2009)
NR2A	Forward 5′-TGGACGTGAACGTGGTAGC-3′ Reverse 5′-CCCCCATGAATGCCCAAGAT-3′	196	Stepulak et al. (2009)
NR2B	Forward 5′-TTCCGTAATGCTCAACATCATG-3′G Reverse 5′-TGCTGCGGATCTTGTTTACAAA-3′	104	Stepulak et al. (2009)
GluR1	Forward 5′-GGTCTGCCCTGAGAAATCCAG-3′ Reverse 5′-CTCGCCCTTGTCGTACCAC-3′	101	Stepulak et al. (2009)
GluR2	Forward 5′-TGTTGGAGTCCACGATGAACG-3′ Reverse 5′-GCAAGATTTACTGGGGTTCCTAA-3′	139	Stepulak et al. (2009)
GluR3	Forward 5′-CGAGAGGGGTGTATGCCATC-3′ Reverse 5′-GAAGCTAGGCGTAACAAAGGAT-3′	104	Stepulak et al. (2009)
GluR4	Forward 5′-ATTGGTGTCAGCGTGGTCTTA-3′ Reverse 5′-CCAGGGAAAACCAGAGGCT-3′	145	Stepulak et al. (2009)
GluR5	Forward 5′-ACTCAGGATCGGAGGGATTTT-3′ Reverse 5′-GGTGACTGCAAACTTGAAAGC-3′	82	Stepulak et al. (2009)
GluR6	Forward 5′-AGCGTCGGTTAAAACATAAGCC-3′ Reverse 5′-GTTTCTTTACCTGGCAACCTTCT-3′	103	Stepulak et al. (2009)
KAR1	Forward 5′-TGAGGATCGCTGCTATCTTGG-3′ Reverse 5′-CGTACTCGCTGTCTCTGAGAA-3′	159	Stepulak et al. (2009)
KAR2	Forward 5′-GATCAACGGGATCATCGAGGT-3′ Reverse 5′-GTGTCCGTGGTCTCGTACTG-3′	93	Stepulak et al. (2009)
mGluR1	Forward 5′-CAGCCGATTCGCTTTAGCC-3′ Reverse 5′-GGGATCGCGGTACTGAAGTTG-3′	248	Stepulak et al. (2009)
GlyR α1	Forward 5′-ACCACTGTGCTCACCATGAC-3′ Reverse 5′-GAACACAAAGAGCAGGCAAA-3′	114	Neumann et al. (2004)
GlyR β	Forward 5′- ATTCCAACAGCAGCAAAGCGAA-3′ Reverse 5′-CACAGATTGGCATTTATGAAA -3′	177	Neumann et al. (2004)
$GABA_A$ α1	Forward 5′-GAAGAGAAAGATTGGCTACTTTGTTATTCAAACAT-3′ Reverse 5′-GAGCGTAAGTGTTGTTTTTCTTAATAAGAGGAT-3′	371	Mizuta et al. (2008)
$GABA_A$ γ1	Forward 5′-CTTTCCCATGGATGAACATTCCTGTCCACTGGAATTTT-3′ Reverse 5′-CAGGCACTGCATCTTTATTGATCCAAAAAGACACCC-3′	320	Mizuta et al. (2008)
PBR	Forward 5′-AGGGTCTCCGCTGGTACGCC-3′ Reverse 5′-TGGGGCAACCTCTGAAGCTC-3′	517	Lee et al. (2004)
GAT1	Forward 5′- GCATCATCTCCTACCTGATCGGTCTCTCTAACATCACTC-3′ Reverse 5′- GCC CGC CAC AAT GAT TGG TGT GAAGAA AG-3′	248	Zaidi et al. (2010)
GAT2	Forward 5′- GGG CCT GCT GTT TCT TCT TCA TGGT-3′ Reverse 5′- TAA ACC CAA GCC ACA CAG AGG GACT-3′	289	Zaidi et al. (2010)
GAT3	Forward 5′- CTTGTCTGTTATCTCCTATTTTCTGGGCCTCGTGATGTTA-3′ Reverse 5′- GTCGTCAACTTCTGGAGTTTCTCGGGCAGTGT-3′	441	Zaidi et al. (2010)
GAT4/BGT1	Forward 5′- GGA TGG ATG CGG GCA CCC AGA-3′ Reverse 5′- CTCATGAAGCCCAGGATGGAGAAGACAACAAA-3′	184	Zaidi et al. (2010)
GAPDH	Forward 5′- CCATGTTCGTCATGGGTGTGAACCA-3′ Reverse 5′- GCCAGTAGAGGCAGGGATGATGTTC-3′	251	Lee et al. (2010a)

astrocytes from up to the fifth passage were used in the study.

Messenger RNA Analysis by Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from human astrocytes and microglia using TRIzol (GIBCO-BRL, Gathersburg, MD). Cells (10^6 cells) were lysed with TRIzol solution and incubated at room temperature for 1 h. The lysates were centrifuged at 10,000 rpm for 10 min and supernatants transferred to new tubes. The purity and amount of the RNA was measured spectrophotometrically. Total RNA (20 μg) was used to synthesize the first strand complementary DNA (cDNA) using Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO-BRL). The cDNA products were then amplified by PCR using a GeneAmp thermal cycler (Applied Biosystems, Foster City, CA). Specific sense and antisense primers for the experiments (Lee et al., 2004, 2010a; Mizuta et al., 2008; Neumann et al., 2004; Stepulak et al., 2009; Zaidi et al., 2010) are listed in Table 1. PCR conditions were as follows: initial denaturation at 95°C for 6 min followed by a 30-cycle amplification program consisting of denaturation at 95°C for 45 s, annealing at 55–60°C for 1 min and extension at 72°C for 1 min. A final extension was carried out at 72°C for 10 min. The amplified PCR products were identified using 1.5% agarose gels containing ethidium bromide (final concentration 0.5 μg mL^{-1}) and visualized under ultraviolet light.

Cell Culture Protocols and Measurements of Intracellular or Extracellular GABA Concentration

For experiments with untreated astrocytes or microglia, cells were incubated overnight in 24 well plates (10^6 cells per well) in 1 mL of DMEM/F12 medium. The medium was aspirated, and fresh medium added. After suitable periods of incubation, the medium was again aspirated and transferred to ELISA plates for measurement of extracellular GABA. To the wells, 0.5 mL of 0.1M potassium phosphate buffer (pH 7.4) was added, and the cells detached by agitation. The mixture was then transferred to a homogenizer. After homogeniza-

tion, the debris was removed by centrifugation at 2,000 rpm for 10 min. The supernatants were transferred to 96 well plates for ELISA analysis. GABA concentrations were measured with detection kits (IBL, Minneapolis, MN) following protocols described by the manufacturer.

For experiments involving stimulation or inhibition of astrocytic receptors, astrocytes were incubated in fresh medium to which the appropriate agents were added. In some experiments, the GABA-T inhibitors gabaculine (1 μM, ICN Pharmaceuticals, Irvine, CA, Behar and Boehm, 1994) or vigabatrin (300 μM, Sigma, Yang et al., 2009) were added with the stimulants. Intracellular and extracellular GABA levels were measured for periods varying from 1 min to 6 h by the methods previously described.

To investigate the effects of individual GABA transporters on GABA release, astrocytes were exposed to media containing one of the following transport inhibitors: NNC 711 (1-(2-(((diphenylmethylene)amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid hydrochloride) at 20 μM for GAT1; β-alanine at 300 μM for GAT2; (S)SNAP-5114 ((*S*)-1-[2-[tris(4-methoxyphenyl)methoxy]ethyl]-3-piperidinecarboxylic acid) at 10 μM for GAT3; and NNC 05-2090 (1-[3-(9*H*-carbazol-9-yl)propyl]-4-(2-methoxyphenyl)-4-piperidinol hydrochloride) at 40 μM for BGT1. Experiments were performed with and without gabaculine or vigabatrin for periods of 1 min to 6 h. Extracellular GABA levels were measured.

To investigate the effects of glutamate, astrocytes were exposed to media to which glutamic acid in concentrations from 0.01 to 300 μM had been added. Experiments were conducted with and without gabaculine or vigabatrin for periods varying from 1 min to 6 h. Extracellular GABA levels were measured.

To investigate the relative effects of specific glutamate receptor blockers, astrocytes were exposed to media containing one of the following inhibitors: glutamate 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) at 30 μM (for AMPA and kainate receptors), MK-801 at 20 μM for NMDA receptors, or CPCCOEt (CPC) at 100 μM for mGluR1 receptors. After 1 h, 50 μM glutamate plus vigabatrin or gabaculine was added to the medium and incubations carried out for 6 h. Extracellular GABA levels were measured.

To explore the effects of NMDA receptor co-agonists, glycine (100 μM) or D-serine (100 μM) were added to the media with or without gabaculine or vigabatrin, and the astrocytes incubated for 6 h. To explore the effects of benzodiazepine receptors, diazepam (0.01–50 μM) was used as an agonist of the "central" benzodiazepine receptor, which involves glycine receptor subtypes, and PK11195 (100 μM) as an agonist of the peripheral benzodiazepine receptors. These agents were added to the media along with gabaculine or vigabatrin and incubations carried out for 6 h. Extracellular GABA levels were then measured.

To investigate the effects of GABA receptor agonists, muscimol (0.01–300 μM) was used as an agonist of $GABA_A$ receptors, and baclofen (0.01–300 μM) as an agonist of $GABA_B$ receptors. They were added to the media with and without gabaculine or vigabatrin. Incubations were carried out for 6 h. Extracellular GABA levels were then measured.

To investigate the extent of GABA release following depolarization, astrocytes were exposed to media containing 100 mM K^+. They were incubated for 6 h with and without vigabatrin and gabaculine. Extracellular GABA levels were then measured.

To investigate whether GABA release is Ca^{2+}-dependent, astrocytes were treated with the cell-permeable Ca^{2+}-chelator, BAPTA-AM (300 μM, Tsien, 1981) for 1 h and were then exposed to 100 mM K^+, 50 μM glutamate, 100 μM glycine, 100 μM D-serine, or 100 μM PK11195 in the presence of the GABA-T inhibitors gabaculine and vigabatrin for 6 h. The cell-free medium was collected to measure GABA levels.

To explore whether GAT-mediated release is Ca^{2+}-dependent, astrocytes were pre-exposed to 300 μM of the $[Ca^{2+}]_i$ depleting agent BAPTA-AM for 1 h and subsequently to NNC 711 (20 μM), β-alanine (300 μM), and/or (S)SNAP-5114 (10 μM) plus either of the GABA-T inhibitors, gacaculine, or vigabatrin for 6 h, and extracellular GABA levels were measured. Conversely, astrocytes were exposed to 500 nM of the $[Ca^{2+}]_i$ enhancing agent ionomycin plus NNC 711 (20 μM), β-alanine (300 μM), and/or (S)SNAP-5114 (10 μM) for 6 h, and extracellular GABA levels were measured. To inhibit GABA-T, gabaculine at 1 μM or vigabatrin at 300 μM, 6 h incubation with ionomycin plus the inhibitors, was used.

To examine circumstances where intracellular Ca^{2+} is involved in GABA release from astrocytes, cells were treated with 2-APB (100 μM), an inhibitor of IP_3 receptor, for 1 h and subsequently were exposed to 100 mM K^+, 50 μM glutamate, 100 μM glycine, 100 μM D-serine, or 100 μM PK11195 in the presence of the GABA-T inhibitors gabaculine or vigabatrin for 6 h. Extracellular GABA was then measured.

Measurement of $[Ca^{2+}]_i$ in Human Astrocytes

A fluorometric measurement of $[Ca^{2+}]_i$ with Fluo-3/AM (Anaspec, CA) as an indicator of Ca^{2+} was performed as described previously (Chen et al., 2008; Paredes et al., 2008). After human astrocytes (5×10^5 cells) were incubated with DMEM/F12 medium containing 10% FBS and the stimulants listed above for 1 min to 6 h, they were exposed to Fluo-3/AM (4 μg/ml) and pluronic F127 (5 μM, Sigma, St. Louis, MO) for 1 h. The cells were washed three times with PBS and the fluorescence emitted from Ca^{2+}-bound Fluo-3 recorded at an excitation of 485 nm and an emission of 526 nm using a fluorescent plate reader (Spectra MAX GeminiXS, Molecular Devices). Absolute values for $[Ca^{2+}]_i$ were calculated according to the equation: $[Ca^{2+}]_i = K_d \times [(F - F_{min})/(F_{max} - F)]$. The F_{max} and F_{min} values were determined by treating cells with 2 μg mL^{-1} ionomycin (Calbiochem, La Jolla, CA) and 10 mM EGTA, respectively. The K_d value of Fluo-3 was previously determined to be 400 nM (Minta et al., 1989).

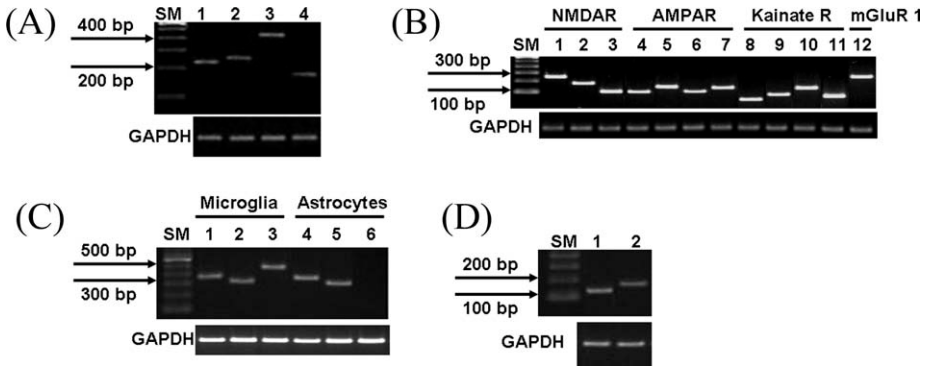

Fig. 1. (**A**) Expression of four isoforms of GABA transporter (GAT1-3, BGT1) mRNA in astrocytes. SM: size markers, 1: GAT1, 2: GAT2, 3: GAT3 and BGT1. Three independent experiments were performed and this is a representative. Note that astrocytes express the mRNAs for all four GABA transporters. (**B**) Expression of subunits for NMDA receptors (NR1, NR2A, NR2B) lanes 1–3; AMPA receptors (GluR1, GluR2, GluR3, GluR4) lanes 4–7; kainate receptors (GluR5, GluR6, KAR1, KAR2) lanes 8–11; and metabotropic glutamate receptor (mGluR1) in human astrocytes. SM: size markers. Three independent experiments were performed and this is a representative. (**C**) Expression of subunits for central and peripheral benzodiazepine receptor mRNA in microglia and astrocytes. SM: size markers, 1 and 4: $GABA_A$ α1 subunit, 2 and 5: $GABA_A$ γ1 subunit, 3 and 6: peripheral benzodiazepine receptor. Three independent experiments were performed and this is a representative. Note that both microglia and astrocytes express central benzodiazepine receptor (CBR) binding sites (α1 and γ1 subunits in $GABA_A$ receptor) but only microglia express PBR mRNA. (**D**) Expression of subunits for glycine receptor mRNA in astrocytes. SM: size markers, 1: GlyR α1 subunit, 2: GlyR β subunit. Three independent experiments were performed and this is a representative. Note that astrocytes express α1 and β subunits mRNAs for functional glycine receptors. GAPDH loading controls are shown in the lower panel.

Cell Viability Assays

The viability of astrocytes following 7-h incubation with all the agents used in these experiments was evaluated by the MTT assay (Lee et al., 2010). There was no difference in viability from astrocytes incubated with fresh medium only (data not shown).

Western Blotting

Western blotting on cell lysates was performed as described by Lee et al. (2011). Briefly, cells were treated with a lysis buffer (150 mM NaCl, 12 mM deoxycholic acid, 0.1% Nonidet P-40, 0.1% triton X-100, and 5 mM Tris-EDTA, pH 7.4). The protein concentration of the cell lysates was then determined using a BCA protein assay reagent kit (Pierce, Rockford, IL). Equal amounts of proteins from each sample were loaded onto gels and separated by 10% SDS-PAGE (150 V, 1.5 h). The loading quantities of lysate proteins for transporter analysis were 100 μg. Following SDS-PAGE, proteins were transferred to a PVDF membrane (Bio-Rad, CA) at 30 mA for 2 h. The membranes were blocked with 5% milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h and incubated overnight at 4°C with a polyclonal anti-GAT1 antibody (Ab64645, Abcam, Cambridge, MA, 1/2,000), a polyclonal anti-GAT2 antibody (NB100-1872, Nobus Biologicals, Littleton, CO, 1/2,000), a monoclonal anti-GAT3 antibody (H00006538-M10, Ab nova, Walnut, CA, 1/2,000) or a polyclonal BGT1 antibody (HPA034973, Sigma, 1/2,000). The membranes were then treated with a horseradish peroxidase-conjugated anti-rabbit IgG (P0448, DAKO, Mississauga, Ontario, CA, 1/2,000) or a horseradish peroxidase-conjugated anti-mouse IgG (A3682, Sigma, 1/3,000) for 3 h at room temperature, and the bands visualized with an enhanced chemiluminescence system and exposure to photographic film (Hyperfilm ECL™, Amersham Pharmacia Biotech, Little Chalfont, UK). Equalization of protein loading was assessed independently using tubulin as the housekeeping protein. The primary antibody was anti-α-tubulin (T6074, Sigma, St Louis MO, 1:2,000) and the secondary antibody anti-mouse IgG (A3682, Sigma, 1:3,000). Primary antibody incubation was overnight at 4°C and secondary antibody incubation 3 h at room temperature.

Data Analysis

The significance of differences in data sets was analyzed by one-way or two-way ANOVA. Multiple group comparisons were followed where appropriate by a post-hoc Bonferroni test.

RESULTS

To determine what molecules might be involved in facilitating GABA release from astrocytes, we explored for candidate mRNAs by RT-PCR. Tests for the presence of 21 mRNAs were carried out, 20 of which were abundantly expressed by astrocytes. They included 4 GABA transporters, 12 glutamate receptors, 2 GABA receptors, 2 glycine receptors, and 1 peripheral benzodiazepine receptor. The primers used are listed in Table 1 and the results are shown in Fig. 1. Figure 1A shows the results for GABA transporters, 1B for glutamate receptors, 1C for GABA and benzodiazepine receptors, and 1D for glycine receptors, all of which were positive except for the peripheral benzodiazepine receptor which was negative for astrocytes but positive for microglia. In all these experiments, equal loading of lanes was demonstrated

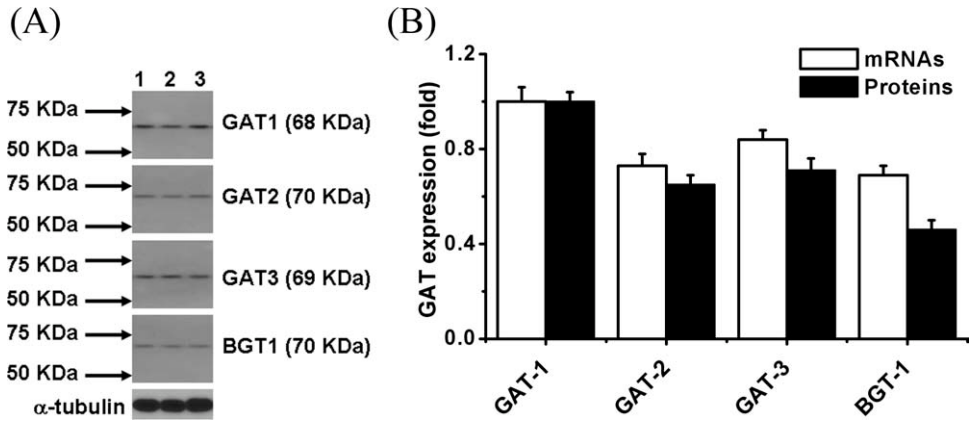

Fig. 2. (**A**) Protein expression of GAT1-3 and BGT1 using Western blotting. The loading quantities of lysate proteins were 100 μg. Each lane represents the three independent experiments. Equalization of protein loading was assessed independently using α-tubulin as the housekeeping protein. (**B**) Quantitative results. For mRNA and protein data, the density of GAT2-3, BGT1 protein band was compared with that of GAT1. Values are mean ± SEM, $n = 3$. See Materials and Methods for experimental details.

TABLE 2. Alterations in Intracellular/Extracellular GABA Concentration in Astrocytes in Time[a]

Time	Extracellular GABA (μmol mL^{-1})	Intracellular GABA (μmol mL^{-1})
0	UD	2.23 ± 0.27
1 min	0.08 ± 0.02	2.16 ± 0.22
10 min	0.13 ± 0.04	2.32 ± 0.27
30 min	0.44 ± 0.09	2.37 ± 0.24
1 h	0.68 ± 0.25	2.24 ± 0.32
2 h	0.72 ± 0.26	2.43 ± 0.12
4 h	0.68 ± 0.23	2.28 ± 0.26
6 h	0.72 ± 0.19	2.35 ± 0.28

[a]Alterations in intracellular/extracellular GABA concentration in astrocytes.
Time: 0 means the time of medium change; UD: Undetectable.
Values are mean ± SEM, $n = 4$.

by comparable intensities of the band for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) utilized as the housekeeping gene.

We also examined the GABA transporters for relative protein expression. Protein lysates were collected independently in different cultures and western blotting was performed. The data are shown in Fig. 2. As expected, the four GABA transporter proteins were expressed (Fig. 2A). Equalization of protein loading was assessed independently using α-tubulin as the housekeeping protein. Quantitative results are shown in Fig. 2B. The expression levels of proteins for the four transporters are roughly comparable, with GAT1 being the highest and BGT1 the lowest.

We next measured intracellular and extracellular GABA levels in astrocytic cultures. Baseline levels of GABA were obtained from astrocytes that had been cultured overnight, with the overnight medium being discarded and fresh medium added. The results after culture periods of up to 6 h are shown in Table 2 for untreated astrocytes and Table 3 for treated astrocytes where the GABA-T inhibitors gabaculine (1 μM), or vigabatrin (300 μM) had been added to the fresh medium. GABA-T inhibitor treatment was for the purpose of increasing intracellular GABA levels so that receptor interaction effects could be enhanced.

The intracellular GABA levels of untreated astrocytes averaged 2.32 mM (μmol mL^{-1}) and did not vary over time. Since fresh medium did not contain GABA, GABA was initially undetectable in the extracellular fluid. However it reached a level of ~0.70 mM after the first hour which was maintained over the subsequent 5 h (Table 2). There was a steady state intracellular-extracellular gradient of 3.64 fold.

The intracellular GABA levels of astrocytes treated with gabaculine or vigabatrin increased rapidly in the first few minutes, and then more gradually over time, reaching a level at 6 h of 38–39 mM. Extracellular GABA levels followed a similar pattern, reaching a concentration of 23–24 mM at 6 h. The intracellular–extracellular concentration gradient was about 1.65 fold, indicating enhanced secretion of GABA into the medium when the intracellular concentrations were increased (Table 3). Almost identical results were obtained with the two inhibitors.

Since the astrocytes were otherwise untreated, it could be hypothesized that GABA transporters were involved. To test this possibility, we next examined the effects of inhibitors of GABA transport systems. We utilized as inhibitors NNC 711 at 20 μM for GAT1 (Borden et al., 1994a), β-alanine at 300 μM for GAT2 (Christiansen et al., 2007; Zaidi et al., 2010), (S)SNAP-5114 at 10 μM for GAT3 (Borden et al., 1994b), and NNC 05-2090 at 40 μM for BGT1 (Zaidi et al., 2010). It must be noted that these inhibitors are not absolutely specific but only preferential for particular transporters. The astrocytes were incubated for 6 h with these inhibitors along with gabaculine or vigabatrin. The results are shown in Fig. 3. Inhibitors for GAT1, GAT2, GAT3, but not BGT1, reduced GABA release from astrocytes by 20–25% compared with gabaculine or vigabatrin alone ($P < 0.01$). These data indicate that GAT1, 2 and 3, but not BGT1, which is otherwise known as the betaine transporter, are involved in maintaining intracellular GABA at homeostatic levels by participating in the export of excess GABA.

TABLE 3. Alterations in Extracellular/Intracellular GABA Levels in Human Primary Cultured Astrocytes in the Presence of Gabaculine or Vigabatrin

	Extracellular GABA levels (μmol mL^{-1})		Intracellular GABA levels (μmol mL^{-1})	
Time	Gabaculine (1 μM)	Vigabatrin (300 μM)	Gabaculine (1 μM)	Vigabatrin (300 μM)
0	0.78 ± 0.13	0.80 ± 0.18	2.24 ± 0.19	2.18 ± 0.22
1 min	3.49 ± 0.37	3.51 ± 0.32	9.92 ± 0.48	9.73 ± 0.26
10 min	9.36 ± 0.88	9.42 ± 0.79	15.56 ± 1.13	16.02 ± 1.31
30 min	12.61 ± 1.45	13.04 ± 0.93	21.08 ± 1.74	22.04 ± 1.61
1 h	16.12 ± 1.52	15.89 ± 1.61	26.51 ± 2.22	27.44 ± 2.05
2 h	18.08 ± 1.85	19.03 ± 1.44	32.95 ± 2.95	33.78 ± 2.27
4 h	21.74 ± 1.59	22.58 ± 1.38	35.95 ± 3.33	36.88 ± 3.16
6 h	23.04 ± 1.84	24.13 ± 1.72	38.26 ± 3.96	39.15 ± 3.03

Values are mean ± SEM, $n = 4$.

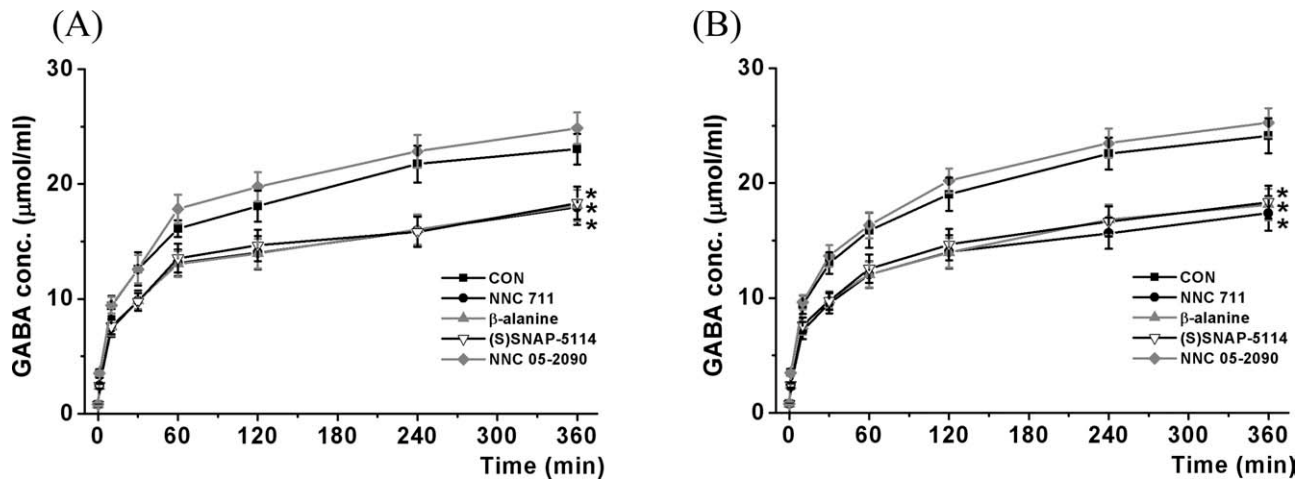

Fig. 3. Effects of pre-treatment with inhibitors for GAT on amount of GABA released from astrocytes after 1–360 min. The inhibitors are NNC 711 (20 μM) for GAT1, β-alanine (300 μM) for GAT2, (S)SNAP-5114 (10 μM) for GAT3, and NNC 05-2090 (40 μM) for BGT1. (**A**) Gabaculine (1 μM) and (**B**) Vigabatrin (300 μM) were used to block GABA-T. Values are mean ± SEM, $n = 4$. Significance of differences was tested by two-way ANOVA. Multiple comparisons were followed with *post-hoc* Bonferroni test where necessary. *$P < 0.01$ for NNC 711, β-alanine, and (S)SNAP-5114 groups compared with the control group between 10 and 360 min.

Since glutamic acid is the most abundant amino acid in brain, as well as being the major excitatory neurotransmitter, we next investigated the effects of glutamate itself, and blockers of its specific receptors, on GABA levels. The astrocytes were treated with the GABA-T inhibitors gabaculine (1 μM) or vigabatrin (300 μM) in some experiments to increase GABA intracellular levels so that receptor effects could be enhanced.

Figure 4A demonstrates GABA release results from astrocytes exposed to media containing 0.01–300 μM glutamate. Without GABA-T inhibitor treatment, glutamate added in the concentration range of 0.01–10 μM did not increase GABA release. This might be expected since the medium itself contains 50 μM glutamate. However, astrocytes did significantly release GABA on treatment with 30 μM added glutamate (total 80 μM), reaching a maximum at 100–300 μM added. When intracellular GABA levels were increased by treatment with GABA-T inhibitors, astrocytes significantly released GABA with as little as 10 μM added glutamate. Again, extracellular GABA levels were dose-dependently increased to reach a maximum at 100–300 μM added glutamate ($P < 0.01$). Almost identical results were obtained with the two GABA-T inhibitors. The ED_{50} values were 51.46 ± 2.56 μM for gabaculine, and 53.27 ± 2.27 μM for vigabatrin.

We next investigated whether individual glutamate receptors, including NMDARs, AMPARs, KARs, and mGluR1 are involved by employing their specific antagonists (20 μM MK-801 for NMDARs, 30 μM NBQX for AMPARs/KARs, and 100 μM CPCCOEt for mGluR1). After exposure of astrocytes to the inhibitors for 1 h, 50 μM glutamate plus gabaculine or vigabatrin was then added to the medium. The results on GABA release are shown in Fig. 4B. Pre-exposure of cells to each of these antagonists for 1 h, reduced the amount of GABA release induced by 50 μM glutamate by 25–30% ($P < 0.01$). These data indicate that each class of glutamate receptors expressed by astrocytes is involved in enhancing GABA release and that inhibition of them reduces such release.

Next we investigated whether receptors for glycine, D-serine, and glutamate could be involved in GABA release. The results are recorded in Table 4. We found that extracellular GABA levels increased after treatment with 100 μM glycine or D-serine but not as much as those observed after treatment with 50 μM glutamate. These data suggested that glycine and D-serine

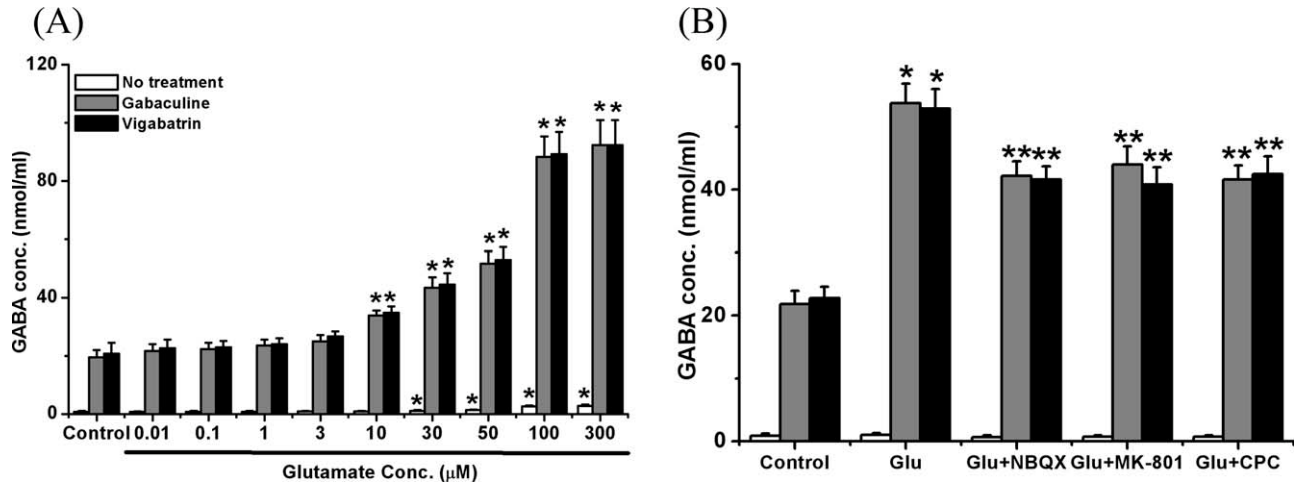

Fig. 4. (**A**) Amount of GABA released from astrocytes after 6 h induced by various concentrations of glutamate. Gabaculine (1 μM) and vigabatrin (300 μM) were used to block GABA-T. Values are mean ± SEM, $n = 4$. Significance of differences was tested by two-way ANOVA. Multiple comparisons were followed with *post-hoc* Bonferroni test where necessary. $^*P < 0.01$ compared with the control group under the same conditions. Note that glutamate increased GABA release in a dose-dependent manner. Glutamate concentrations inducing a half of maximal release (ED_{50}) are 51.46 ± 2.56 μM for gabaculine and 53.27 ± 2.27 μM for vigabatrin. (**B**) Effects of treatment with glutamate antagonists on release of GABA induced by 50 μM glutamate. The antagonists were NBQX (30 μM, an antagonist for KARs/AMPARs), MK-801 (20 μM, an antagonist for NMDARs) or CPCCOEt (CPC, 100 μM, an antagonist for mGluR1). Gabaculine (1 μM) and Vigabatrin (300 μM) were used to block GABA-T. Values are mean ± SEM, $n = 4$. Significance of differences was tested by one-way ANOVA. $^*P < 0.01$ for Glu group compared with the control group under the same conditions and $^{**}P < 0.01$ for Glu+NBQX, Glu+MK-801 or Glu+CPC group compared with Glu group under the same conditions.

TABLE 4. Extracellular GABA Concentration in Astrocytes treated with 50 μM Glutamate, 100 μM Glycine, or 100 μM D-Serine

	GABA levels (μmol mL^{-1})					
	Gabaculine (1 μM)			Vigabatrin (300 μM)		
Time	Glutamate (50 μM)	Glycine (100 μM)	D-Ser (100 μM)	Glutamate (50 μM)	Glycine (100 μM)	D-Ser (100 μM)
0	0.82 ± 0.13	0.76 ± 0.24	0.82± 0.21	0.78 ± 0.22	0.79 ± 0.15	0.80 ± 0.21
1 min	6.66 ± 0.88	4.05 ± 0.22	3.95 ± 0.35	7.34 ± 0.83	4.13 ± 0.15	4.15 ± 0.23
10 min	12.88 ± 2.66	9.87 ± 0.57	10.03 ±0.46	13.34 ± 2.66	9.95 ± 0.62	8.93 ± 0.44
30 min	18.52 ± 3.15	11.42 ± 0.83	11.56 ± 0.63	19.11 ± 2.15	12.02 ± 0.72	12.66 ± 0.81
1 h	26.72 ± 3.66	19.34 ± 1.32	18.55 ± 0.72	27.73 ± 2.66	18.68 ± 0.78	17.93 ± 1.13
2 h	36.88 ± 3.68	27.28 ± 1.53	26.88 ± 0.63	35.99 ± 3.15	26.66 ± 1.73	27.44 ± 1.48
4 h	44.61 ± 4.12	34.72 ± 1.83	33.72 ± 2.01	43.92 ± 3.77	35.66 ± 1.95	32.74 ± 2.17
6 h	53.19 ± 4.34	39.06 ± 2.55	40.62 ± 2.85	52.65 ± 4.26	38.49 ± 2.17	39.84 ± 2.37

Human astrocytes were treated with glutamate, glycine (Gly), or D-serine (D-Ser) plus 1 μM gabaculine or 300 μM vigabatrin for 1–360 min and their supernatants were collected to measure the amount of released GABA using ELISA assays. Values are mean ± SEM, $n = 4$. Significance of differences was tested by two-way ANOVA. Multiple comparisons were followed with *post-hoc* Bonferroni test where necessary. Note that $P < 0.01$ for Gly or D-Ser group compared with glutamate group from 1 to 360 min.

induce GABA release by weakly activating NMDARs as co-agonists (Boehning and Snyder, 2003; Haydon and Carmignoto, 2006).

We then explored the role of GABA receptor sites. We have previously reported that astrocytes express the mRNA and proteins for $GABA_A$ and $GABA_B$ receptors both *in vitro* and *in vivo* (Lee et al., 2011). To test their involvement in GABA release, we used muscimol as an agonist for the $GABA_A$ receptor and baclofen as an agonist for the $GABA_B$ receptor. It is reported that diazepam binds to α-γ subunits of $GABA_A$ receptors (Johnston, 1996) so diazepam was also tested. The tests were run on untreated as well as gabaculine and vigabatrin treated astrocytes. The results are shown in Fig. 5. Muscimol treatment at 1–300 μM resulted in an ~3-fold increase in GABA release in gabaculine and vigabatrine treated astrocytes, reaching its maximum at 100 μM (Fig. 5A, ED_{50}s: 8.47 ± 1.33 μM for gabaculine and 9.15 ± 1.41 μM for vigabatrin). Significant but slightly lower increases in GABA release were obtained after treatment with baclofen as the $GABA_B$ receptor stimulant (Fig. 5B) ($P < 0.01$, ED_{50}s: 15.47 ± 1.44 μM for gabaculine and 18.36 ± 1.37 μM for vigabatrin. The most powerful agonist was diazepam (Fig. 5C, ED_{50}s: 2.25 ± 0.22 μM for gabaculine and 2.72 ± 0.27 μM for vigabatrin). These data indicate that stimulation of both $GABA_A$ and $GABA_B$ receptors results in GABA release with the diazepam binding sites being the most sensitive. However, treatment of human microglia with diazepam at the same concentrations did not show any GABA release (data not shown) because the cells do not have GAD proteins and therefore do not produce GABA (Lee et al., 2011).

PK11195 treatment of astrocytes failed to increase either intracellular or extracellular GABA levels, but this would not be anticipated since astrocytes were shown do

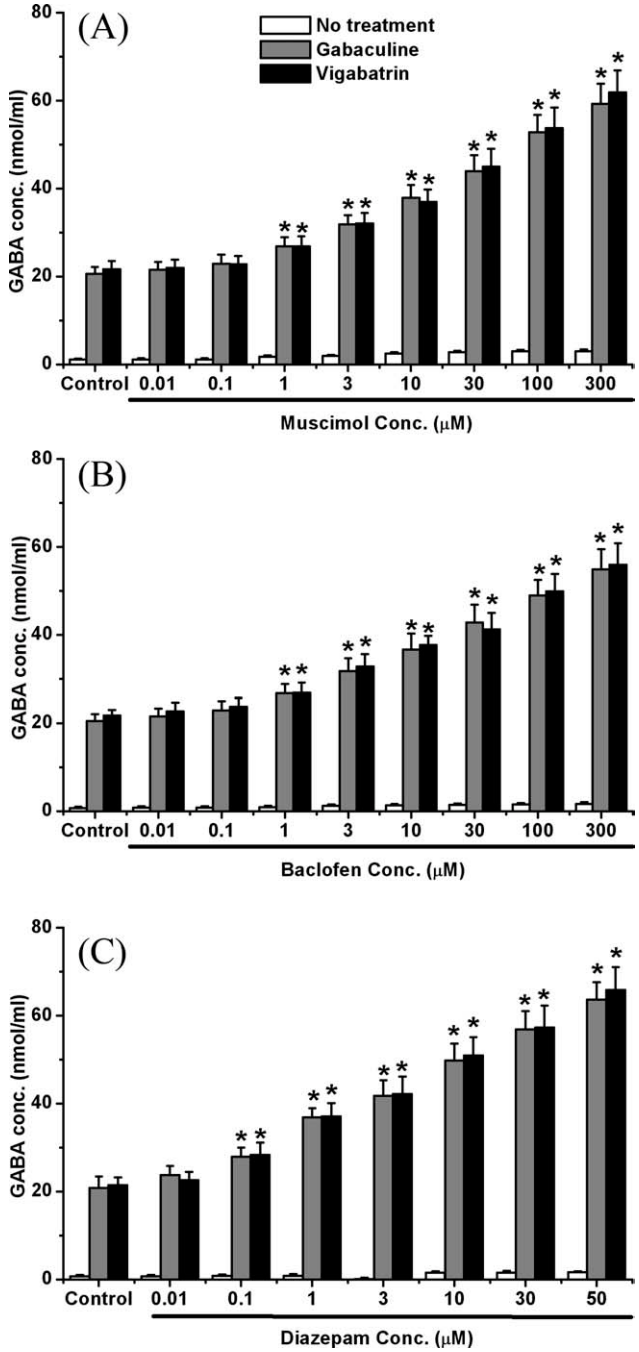

Fig. 5. Amount of GABA released from astrocytes after 6 h induced by various concentration of muscimol (**A**) or baclofen (**B**) or diazepam (**C**). Gabaculine (1 μM) and vigabatrin (300 μM) were used to block GABA-T. Values are mean ± SEM, $n = 4$. Significance of differences was tested by two-way ANOVA. Multiple comparisons were followed with *post-hoc* Bonferroni test where necessary. *$P < 0.01$ compared with control group in the same condition. Note that diazepam and muscimol, an agonist for $GABA_A$ receptors, and baclofen, an agonist for $GABA_B$ receptors, increased GABA release in a dose-dependent manner. ED_{50}s are (**A**) 12.47 ± 1.33 μM (gabaculine) and 13.15 ± 1.41 μM (vigabatrin) for muscimol, (**B**) 15.47 ± 1.44 μM (gabaculine) and 16.36 ± 1.37 μM (vigabatrin) for baclofen; and (**C**) 2.25 ± 0.22 μM (gabaculine) and 2.72 ± 0.27 μM (vigabatrin) for diazepam.

TABLE 5. Alterations in Extracellular GABA Concentration in Human Primary Cultured Astrocytes by Treatment with 100 mM K^+ in the Presence of 1 μM Gabaculine or 300 μM Vigabatrin

	GABA levels (μmol mL^{-1})			
	Gabaculine (1 μM)		Vigabatrin (300 μM)	
Time	No K^+	100 mM K^+	No K^+	100 mM K^+
0	0.81 ± 0.22	0.79 ± 0.12	0.78 ± 0.15	0.77 ± 0.21
1 min	3.51 ± 0.28	6.73 ± 0.95	3.47 ± 0.25	6.95 ± 0.36
10 min	9.42 ± 0.75	14.89 ± 1.44	9.58 ± 0.82	15.66 ± 1.74
30 min	13.93 ± 1.24	18.74 ± 1.32	13.27 ± 1.02	19.48 ± 2.15
1 h	17.22 ± 1.31	26.39 ± 2.01	16.03 ± 1.40	27.11 ± 2.14
2 h	19.33 ± 1.74	34.36 ± 2.35	20.26± 1.62	33.96 ± 1.62
4 h	22.15 ± 1.27	42.66 ± 3.04	23.44± 1.48	41.55 ± 2.16
6 h	24.55 ± 1.69	51.93 ± 3.27	25.43 ± 1.61	52.55 ± 2.85

Human astrocytes were treated with 100 mM K^+ for 1 min to 6 h and their supernatants were collected to measure the amount of released GABA using ELISA assays. To inhibit GABA-T gabaculine at 1 μM or vigabatrin at 300 μM was used. Values are mean ± SEM, $n = 4$. Significance of differences was tested by two-way ANOVA. Multiple comparisons were followed with *post-hoc* Bonferroni test where necessary. Note that there was a significant increase in GABA levels for 100 mM K^+ group compared with untreated group from 1 min to 6 h in both gabaculine and vigabatrin treated cells.

not express the mRNA or protein for this receptor (see Table 1 and Fig. 1).

To assess the effects of simple depolarization of astrocytes compared with stimulation of specific receptors, astrocytes treated with 100 mM K^+ were incubated for periods of 1 min to 6 h with and without vigabatrin and gabaculine. The results are shown in Table 5. Extracellular GABA levels were dramatically increased in as short a period as 1 min and continuously increased to reach 51–52 mM.

To determine whether influx of Ca^{2+} ions is involved in the receptor-stimulated release of GABA from astrocytes, we examined the intracellular accumulation of Ca^{2+} by employing Fluo-3/AM as the measuring tool. Astrocytes were cultured for 1 h with or without the calcium chelator BAPTA-AM (300 μM) and then 50 μM glutamate, 100 μM glycine, 100 μM D-serine, 100 μM PK11195 or 100 mM K^+, along with 300 μM vigabatrin was added and incubation carried out for the following 6 h. The results are shown in Table 6. Without treatment, astrocytes had $[Ca^{2+}]_i$ levels of around 65–70 nM. Treatment with 50 μM glutamate or 100 mM K^+ induced an influx of Ca^{2+} so that the $[Ca^{2+}]_i$ was dramatically increased after 1 min, and then progressively increased up to 6 h where levels reached 310–330 nM ($P < 0.01$). Glycine and D-serine also increased $[Ca^{2+}]_i$ to 260–280 nM ($P < 0.01$),but these were significantly lower than with glutamate ($P < 0.01$). There was no increase in $[Ca^{2+}]_i$ in the presence of PK11195.

To determine the effects of this Ca^{2+} influx, experiments were carried out where BAPTA-AM was added to the medium. BAPTA-AM is rapidly taken up by cells and converted intracellularly into the calcium chelator BAPTA. BAPTA-AM reduced the free intracellular Ca^{2+} levels to 30–38 nM in all experiments (Table 6). It produced an inhibition of GABA release upon stimulation of GABA receptors. The results are shown in Table 7. It was found that the release of GABA resulting from stimulation of astrocytic receptors by 100 mM K^+, 50 μM glutamate, 100 μM glycine, or 100 μM D-serine was

TABLE 6. Alterations in Intracellular Ca^{2+} Concentration (nM) Induced by K^+, Glutamate, Glycine, D-Serine or PK11195 in the Presence of BAPTA-AM

	Intracellular Ca^{2+} concentration (nM)											
	Control		100 mM K^+		50 mM glutamate		100 mM glycine		100 mM D-serine		100 mM PK11195	
Time	NO BAPTA	BAPTA	NO BAPTA	BAPTA	NO BAPTA	BAPTA	NO BAPTA	BAPTA	NO BAPTA	BAPTA	NO BAPTA	BAPTA
0	66.86±5.75	67.23±2.55	66.73±3.96	66.83±2.63	67.93±3.11	67.12±3.11	68.34±3.18	65.47±2.95	69.34±2.95	65.37±2.89	65.86±3.55	66.93±3.19
1 min	65.77±4.54	30.45±2.14	269.64±10.99	31.65±2.16	259.32±4.75	30.46±2.12	220.35±4.65	32.77±1.84	201.56±4.77	31.45±2.25	66.14±2.94	32.36±2.54
10 min	65.55±4.15	32.74±1.94	277.75±6.03	32.77±1.89	266.85±5.23	31.65±2.25	230.64±5.22	31.84±2.12	222.57±3.95	32.44±2.36	67.23±3.74	31.33±2.15
30 min	66.83±3.63	30.56±1.84	285.95±8.01	30.45±1.93	276.93±5.83	30.85±2.17	242.83±5.95	32.66±1.69	235.77±4.66	31.76±2.17	66.82±4.01	32.57±2.61
1 h	68.93±4.74	31.45±2.74	294.83±9.77	33.37±1.77	288.53±6.34	32.73±1.94	255.54±6.39	31.58±2.06	248.94±5.17	30.42±1.73	67.91±3.64	31.46±2.55
2 h	70.94±4.71	30.82±1.59	306.51±10.25	35.82±1.48	295.56±7.74	34.97±1.88	268.45±7.77	32.66±1.79	256.95±6.44	31.74±2.16	66.93±2.85	32.55±1.85
4 h	71.84±4.95	31.77±1.77	318.76±11.04	37.13±1.69	302.88±8.83	37.88±2.10	271.94±8.37	30.83±1.85	258.57±6.92	32.66±2.11	68.34±3.36	31.82±1.94
6 h	69.34±3.64	31.96±1.89	330.76±9.11	38.01±1.77	310.33±9.92	37.48±2.15	280.66±8.44	31.95±1.79	260.82±6.16	30.84±1.95	67.92±2.93	30.84±1.69

Cells were pre-exposed to 300 μM BAPTA-AM for 1 h and subsequently to 100 mM K^+, 50 μM glutamate, 100 μM glycine, 100 μM D-serine or 100 μM PK11195 for 1 min to 6 h, and the $[Ca^{2+}]_i$ measured using the Ca^{2+} indicator Fluo-3/AM. To inhibit GABA-T vigabatrin at 300 μM was used. Values are mean ± SEM, $n = 4$. Significance of differences was tested by two-way ANOVA. Multiple comparisons were followed with a *post-hoc* Bonferroni test where necessary. Note that $P < 0.01$ for K^+, glutamate, Gly, D-Ser or PK11195 group compared with untreated (con) group from 1 min to 6 h, $P < 0.01$ for Gly or D-Ser group compared with K^+ or glutamate group from 1 min to 6 h and $P < 0.01$ all the No BAPTA treated groups compared with BAPTA-treated group in the same conditions from 1 min to 6 h.

reduced by 78–84% by pretreatment with BAPTA-AM for 1 h (4–8 μM, $P < 0.01$).

To investigate whether GAT-mediated GABA release was Ca^{2+}-dependent, two sets of experiments were carried out. In one set, BAPT-AM was used to decrease $[Ca^{2+}]_i$. In the other, ionomycin was used to increase $[Ca^{2+}]_i$ (Morgan and Jacob, 1994). In the first set, astrocytes were pre-exposed to 300 μM BAPTA-AM for 1 h and subsequently to NNC 711 (20 μM), β-alanine (300 μM), or (S)SNAP-5114 (10 μM) for 6 h, and extracellular GABA levels were measured. To inhibit GABA-T, gabaculine at 1 μM or vigabatrin at 300 μM for 6 h incubation with GAT inhibitors, was used. The results are shown in Table 8. Pre-exposure of astrocytes to BAPTA-AM resulted in a significant decrease in GABA release (79–83% reduction, $P < 0.01$). Treatment with the inhibitors for GAT1, GAT2, or GAT3 potentiated this reduction in release of GABA. Treatment with the three GAT inhibitors together completely blocked GABA release ($P < 0.01$). These data establish that GAT-mediated release does not depend on $[Ca^{2+}]_i$.

The second set of experiments further confirmed this finding. Astrocytes were exposed to 500 nM ionomycin to increase rather than decrease $[Ca^{2+}]_i$. Ionomycinn was added in addition to NNC 711 (20 μM), β-alanine (300 μM), or (S)SNAP-5114 (10 μM) for 6 h in the presence of gabaculine at 1 μM or vigabatrin at 300 μM. Extracellular GABA levels were measured. The results are shown in Table 9. While treatment with ionomycin significantly increased GABA release ($P < 0.01$), each of the three GAT inhibitors lowered extracellular GABA levels. Treatment with the three GAT inhibitors combined significantly reduced GABA release ($P < 0.01$). The data further indicate that GAT-mediated GABA release is Ca^{2+}-independent.

Finally, we explored further whether Ca^{2+} released from intracellular Ca^{2+} stores could be involved in GABA release from astrocytes. Astrocytes were exposed for 1 h to 2-APB (100 μM, Kukkonen et al., 2001), an inhibitor of the IP_3 receptor, and subsequently to 100 mM K^+, 50 μM glutamate, 100 μM glycine, 100 μM D-serine, or 100 μM PK11195. The results are shown in Fig. 6. The 2-APB reduced GABA release by about one half for all stimulatory treatments ($P < 0.01$).

DISCUSSION

The data presented here provide some insight into the manner in which adult human astrocytes function as GABAergic cells. We showed that *in vitro*, they continuously generated GABA, maintaining a steady state intracellular level of roughly 2.34 mM (Tables 2 and 3). Surplus GABA was released into the surround via GABA transporters in a Ca^{2+}-independent manner, as assessed by lowering $[Ca^{2+}]_i$ with BAPTA-AM and increasing it with ionomycin (Fig. 3 and Tables 8 and 9). The GABA transporter-mediated GABA release from astrocytes may be coupled with glutamate uptake since it has been reported that activation of glutamate

TABLE 7. Effect of Pretreatment with BAPTA-AM (300 μM) on Amount of GABA Released from Astrocytes for 6 h Induced by 100 mM K^+, 50 μM Glutamate, 100 μM Glycine, 100 μM D-Serine, or 100 μM PK11195

	GABA levels (μmol mL^{-1})					
	No treatment		Gabaculine (1 μM)		Vigabatrin (300 μM)	
	NO BAPTA	BAPTA	NO BAPTA	BAPTA	NO BAPTA	BAPTA
Control	0.80 ± 0.21	0.14 ± 0.09	21.33 ± 0.96	4.34 ± 0.55	22.43 ± 1.56	4.84 ± 0.66
100 mM K^+	0.79 ± 0.17	0.13 ± 0.10	48.98 ± 3.89	7.12 ± 1.56	49.53 ± 3.34	7.79 ± 1.34
50 μM glutamate	0.81 ± 0.20	0.15 ± 0.11	49.65 ± 3.34	7.88 ± 1.33	50.93 ± 3.42	8.05 ± 1.27
100 μM glycine	0.82 ± 0.21	0.13 ± 0.10	39.23 ± 3.34	5.02 ± 1.35	40.34 ± 3.58	5.72 ± 1.33
100 μM D-serine	0.79 ± 0.22	0.14 ± 0.07	40.18 ± 4.23	4.92 ± 1.28	41.72 ± 3.72	5.32 ± 1.37
100 μM PK11195	0.81 ± 0.20	0.12 ± 0.06	22.34 ± 2.83	4.12 ± 1.29	23.84 ± 2.66	4.36 ± 1.42

Cells were pre-exposed to 300 μM BAPTA-AM for 1 h and subsequently to 100 mM K^+, 50 μM glutamate, 100 μM glycine, 100 μM D-serine, or 100 μM PK11195 for 6 h, and extracellular GABA levels were measured. To inhibit GABA-T gabaculine at 1 μM or vigabatrin at 300 μM was used. Values are mean ± SEM, $n = 4$. Significance of differences was tested by one-way ANOVA. Multiple comparisons were followed with a *post-hoc* Bonferroni test where necessary. Note that $P < 0.01$ for BAPTA-treated group compared with No BAPTA group in the same condition, $P < 0.01$ for gabaculine or vigabatrin-treated group compared with the untreated group in the same condition, $P < 0.01$ for K^+, glutamate, Gly or D-Ser group compared with untreated (control) group in the same condition and $P < 0.01$ for Gly or D-Ser group compared with K^+ or glutamate group in the same condition.

TABLE 8. Effect on the Amount of GABA Released from Astrocytes 6 h After Treatment with Inhibitors of GAT-1, GAT-2, or GAT-3 Following Pretreatment with BAPTA-AM

	GABA levels (μmol mL^{-1})		
	No treatment	Gabaculine (1 μM)	Vigabatrin (300 μM)
Control (no BAPTA)	0.81 ± 0.16	22.13 ± 1.93	23.06 ± 2.11
BAPTA	0.14 ± 0.02*	4.45 ± 0.63*	4.95 ± 1.11*
BAPTA/NNC 711	0.09 ± 0.01**	2.15 ± 0.22**	1.84 ± 0.33**
BAPTA/β-alanine	0.08 ± 0.02**	2.88 ± 0.17**	1.72 ± 0.33**
BAPTA/(S)SNAP-5114	0.09 ± 0.01**	2.55 ± 0.06**	1.67 ± 0.37**
BAPTA/NNC 711/β-alanine/(S)SNAP-5114	ND	0.24 ± 0.01***	0.13 ± 0.01***

Cells were pre-exposed to 300 μM BAPTA-AM for 1 h and subsequently to NNC 711 (20 μM), β-alanine (300 μM), and/or (S)SNAP-5114 (10 μM) for 6 h, and extracellular GABA levels were measured. To inhibit GABA-T, gabaculine at 1 μM or vigabatrin at 300 μM (6-h incubation with GAT inhibitors) was used. Values are mean ± SEM, $n = 4$. Significance of differences was tested by one-way ANOVA.
*$P < 0.01$ for the BAPTA group compared with the no-BAPTA group;
**$P < 0.01$ for the BAPTA/NNC 711, or the BAPTA/β-alanine, or the BAPTA/(S)SNAP-5114 groups compared with the no-BAPTA group;
***$P < 0.01$ for the BAPTA/NNC 711 plus β-alanine plus (S)SNAP-5114 group compared with BAPTA/NNC 711, or the BAPTA/β-alanine or the BAPTA/(S)SNAP-5114 group.ND, non detected.

TABLE 9. Effect on Amount of GABA Released from Astrocytes After 6 h Following Treatment with Inhibitors of GAT-1, GAT-2, or GAT-3 in the Presence of a Ca^{2+} Ionophore, Ionomycin

	GABA levels (μmol mL^{-1})		
	No treatment	Gabaculine (1 μM)	Vigabatrin (300 μM)
Control	0.77 ± 0.16	24.55 ± 2.93	23.84 ± 1.77
Ionomycin	3.34 ± 0.38*	47.94 ± 1.25*	44.95 ± 1.06*
Ionomycin/NNC 711	3.11 ± 0.17	45.15 ± 1.17	43.86 ± 1.33
Ionomycin/β-alanine	3.13 ± 0.19	45.83 ± 1.17	42.84 ± 1.19
Ionomycin/(S)SNAP-5114	3.14 ± 0.12	46.28 ± 1.06	42.99 ± 1.03
Ionomycin/ NNC 711/β-alanine/(S)SNAP-5114	2.63 ± 0.12**	44.55 ± 0.76**	40.13 ± 1.01**

Cells were exposed to 500 nM ionomycin plus either NNC 711 (20 μM), β-alanine (300 μM), or (S)SNAP-5114 (10 μM) or all three together for 6 h, and the extracellular GABA levels then measured. To inhibit GABA-T, gabaculine at 1 μM or vigabatrin at 300 μM (6 h incubation with ionomycin plus the inhibitors) was used. Values are mean ± SEM, $n = 4$. Significance of differences was tested by one-way ANOVA.
*$P < 0.01$ for the ionomycin group compared with the control group;
**$P < 0.01$ for the ionomycin/NNC 711 plus β-alanine plus (S)SNAP-5114 group compared with the ionomycin group.

transporters results in GABA release from astrocytes (Heja et al., 2009).

We have previously showed that astrocytes and microglia are GABAceptive cells and that GABA inhibits NFκB and P38 MAP kinase activation of microglial pathways (Lee et al., 2011). Thus astrocytes can serve not only to modulate neurotransmitter functioning (Schousboe, 1977, 2003) but also microglial functioning (Lee et al., 2011).

Here we also showed that receptor mediated release of GABA, but not transport mediated release, was accompanied by an increase in $[Ca^{2+}]_i$. Treatment with BAPTA-AM prevented that increase, presumably by BAPTA chelating free $[Ca^{2+}]_i$, thus showing that $[Ca^{2+}]_I$ was actively involved in the release.

The amounts of GABA released by glycine and D-serine stimulation were significantly lower than those released by K^+ and glutamate stimulation. It is known that both glycine and D-serine act as co-agonists of NMDA receptors (Boehning and Snyder, 2003; Haydon and Carmignoto, 2006) as do glycine receptors (Synder and Bennett, 1976).

We found that GABA release induced by high K^+, glutamate, glycine, or D-serine was reduced by prior treatment with 2-APB, a blocker of IP_3 receptors. Such data indicate that GABA release can be associated not only with Ca^{2+} influx from the extracellular milieu, but also Ca^{2+} release from intracellular Ca^{2+} stores by metabotropic receptor activation.

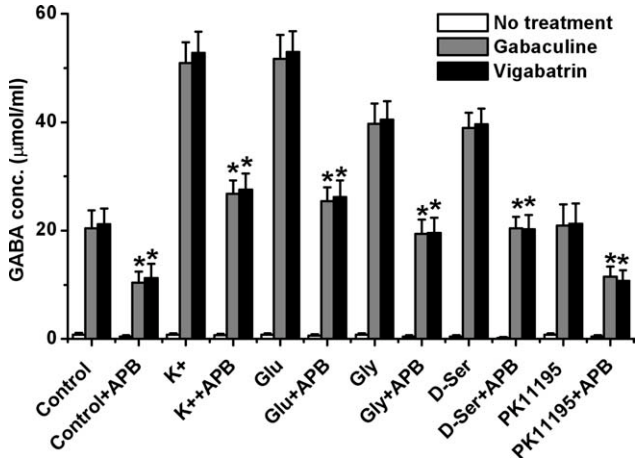

Fig. 6. Effect of pretreatment with the IP_3 receptor antagonist, 2-APB (100 μM for 1 h), for 1 h on amount of GABA released from astrocytes for 6 h induced by 100 mM K^+, 50 μM glutamate, 100 μM glycine, 100 μM D-serine, or 100 μM PK11195. gabaculine (1 μM) and Vigabatrin (300 μM) were used to block GABA-T. Values are mean ± SEM, $n = 4$. Significance of differences was tested by one-way ANOVA. $^*P < 0.01$ for Control +2-APB, K^++2-APB, glutamate +2-APB, Gly +2-APB, or D-Ser +2-APB group compared with control, K+, glutamate, Gly or D-ser group under the same conditions.

As shown in Tables 6 and 7, additional GABA was found to be released in the presence of BAPTA. We showed that this Ca^{2+}-independent pool is governed by GABA transporters (Tables 8 and 9).

Treatment of astrocytes with PK11195, a powerful ligand of the peripheral benzodiazepine receptor (Klegeris et al., 2000), did not cause an increase in $[Ca^{2+}]_i$, nor did it influence intracellular and extracellular GABA levels. Since it was found that this receptor was expressed in microglia, but not astrocytes, this would explain why there were no astrocytic effects of PK11195.

With regard to diazepam, it binds to $GABA_A$ receptors. Historically, the binding of diazepines in brain were referred to as attaching to the central benzodiazepine receptor. This has created confusion in understanding the pharmacology of diazepines and distinguishing their actions from those of PK11195 and analogous molecules. Clarification can be found in reviews by Hertz (Hertz, 1993; Hertz and Chen, 2006).

With regard to activities near the synapse, there are studies indicating that raising astrocyte $[Ca^{2+}]_i$ increases the frequency of excitatory synaptic currents recorded in neighboring neurons which also induce GABA release from inhibitory interneurons in the hippocampus (Kang et al., 1998). Repetitive stimulation of an interneuron evokes release of GABA, which inhibits postsynaptic neurons but also activates astrocyte $GABA_B$ receptors, raising astrocyte $[Ca^{2+}]_i$ to release neurotransmitters such as glutamate (Andersson et al., 2007; Kang et al., 1998). In our investigation, we observed that activation of $GABA_B$ receptors with baclofen induced an increase in $[Ca^{2+}]_i$ which was accompanied by GABA release from astrocytes in a dose-dependent manner. We also observed that activation of $GABA_A$ receptors with muscimol or diazepam induced an increase in $[Ca^{2+}]_i$, which was consistent with previous results (Fraser et al., 1996; Kettenmann and Schachner, 1985; Hertz et al., 2006), and resulted in GABA release from astrocytes. However, its potency in stimulating GABA release was lower than that observed with diazepam (EC_{50}s: 15–18 μM for baclofen vs. 2–3 μM for diazepam), suggesting that the $GABA_A$ receptor is more influential.

Under the conditions of these experiments, where DMEM/F12 medium, which contains 50 μM glutamate and 2.5 mM glutamine was utilized (see invitrogen web page: http://in vitrogen.com), it is difficult to evaluate whether there are conditions under which astrocytes can become glutamate releasers, thus mimicking glutamatergic cells. Microglia, which are GABAceptive cells, can apparently do so. It has been reported that in serum free medium, cultured mouse microglia release glutamate (Patrizio and Levi, 1994), providing a mechanism by which microglia could stimulate GABA release from astrocytes.

With respect to synaptic function, it has been reported that tonic inhibition in the cerebellum is due to astrocyte release of GABA through bestrophin-1 anion channels. Astrocytes have been thought to function as glutamate uptake cells protecting the survival and function of neurons and, through glutamate release, contributing to glutamate receptor activations (Anderson and Swanson, 2000). While astrocytes have been proposed to release "gliotransmitters," the matter is still controversial as reviewed by Hamilton and Atwell (2010).

Regardless of what may take place at synapses, our study clearly demonstrates that GABA is synthesized and released by astrocytes and that they possess multiple receptors designed to facilitate GABA release through Ca^{2+}-dependent and Ca^{2+}-independent mechanisms.

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