Fusion-related release of glutamate from astrocytes

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Summary

Although cell culture studies have implicated the presence of vesicle proteins in mediating the release of glutamate from astrocytes, definitive proof requires the identification of the glutamate release mechanism and the localization of this mechanism in astrocytes at synaptic locales. In cultured murine astrocytes we show an array of vesicle proteins, including SNARE proteins, and vesicular glutamate transporters that are required to fill vesicles with glutamate. Using immunocytochemistry and single-cell multiplex RT-PCR we demonstrate the presence of these proteins and their transcripts within astrocytes freshly-isolated from the hippocampus. Moreover, immunoelectron microscopy demonstrates the presence of VGLUT1 in processes of astrocytes of the hippocampus. To ask whether calcium-dependent glutamate release is mediated by exocytosis we express the SNARE motif of synaptobrevin II to prevent the formation of SNARE complexes, which reduces glutamate release from astrocytes. To further determine whether vesicular exocytosis mediates calcium-dependent glutamate release from astrocytes, we perform whole-cell capacitance measurements from individual astrocytes and demonstrate an increase in whole-cell capacitance, coincident with glutamate release. Together, these data allow us to conclude that astrocytes in situ express vesicle proteins necessary for filling vesicles with the chemical transmitter glutamate and that astrocytes release glutamate through a vesicle- or fusion-related mechanism.

During the past decade there has been increasing evidence for both integrative and dynamic roles for astrocytes in the central nervous system. Following activation of G protein-coupled receptors, astrocytes exhibit calcium oscillations, leading to the release of the chemical transmitters glutamate and ATP (1-3). Studies in vitro and in brain slices have led to the hypothesis of tripartite synaptic transmission (4); neuronal activity causes elevations of synaptically-associated calcium in astrocytes which in turn leads to the release of chemical transmitters from these glial cells to locally modulate synaptic transmission (2;5-
The mechanisms mediating the release of these transmitters from astrocytes are, however, ill-defined and are still the subject of intense debate. At least three distinct release pathways have been proposed as mediating the calcium-dependent release of glutamate from astrocytes: the reversal of plasma membrane glutamate transporters, anion transporter mediate release mechanisms, and calcium-dependent exocytosis (8-10). Because the release of glutamate is stimulated by calcium elevations, is not affected by glutamate transport inhibitors and because changes in cell volume have not been detected coincident with release, it has been proposed that this transmitter is released through a vesicle-mediated exocytotic pathway.

Several observations made using cultured astrocytes support such a vesicle-mediated exocytotic mechanism of glutamate release, including the calcium-dependence of glutamate release (11-13), the presence of vesicle proteins such as synaptobrevin II and syntaxin 1A (13-15) the pulsatile, quantal release of this transmitter that was detected by HEK cells expressing NMDA receptors (16), and the sensitivity of the release process to botulinum and tetanus toxins (17;18). Although significant evidence supports release through such a pathway a recent report has questioned the validity of a vesicular mechanism of glutamate storage and release (9) and proposes instead that release is mediated by a channel through which transmitter is released from the cytosol. Additionally, P2X7 receptor activation has been shown to be capable of mediating the release of cytosolic glutamate through the pore of the channel (19). If the release of glutamate is mediated by an exocytotic mechanism, then astrocytes should express the vesicular glutamate transporters required for the filling of vesicles with glutamate, perturbation of protein complexes known to be essential for exocytosis should block calcium-dependent glutamate release and membrane cycling and, finally, biophysical approaches that can monitor fusion of
vesicles with the plasma membrane should detect an increase in membrane surface area coincident with the release of glutamate.

In this study we have tested each of these possibilities. Because there is significant concern about whether cultured astrocytes accurately represent their \textit{in situ} counterparts, we also asked whether proteins known to be essential for exocytosis are present \textit{in situ} as well as in cell culture. We have determined which vesicle and vesicle-associated proteins are expressed in astrocytes in culture, in astrocytes freshly isolated from the nervous system and in astrocytes \textit{in situ}. After demonstrating the presence of SNARE proteins and vesicular glutamate transporters in freshly-isolated astrocytes and, in the case of VGLUT1, also \textit{in situ}, we then demonstrated the importance of these proteins for glutamate release by perturbing the formation of SNARE protein complexes. Finally, we applied biophysical approaches capable of detecting the fusion of vesicles with the plasma membrane and showed that, coincident with vesicle insertion into the plasma membrane, astrocytes do release the chemical transmitter glutamate. Together, these studies provide strong support to the hypothesis that calcium-dependent glutamate release from astrocytes is mediated by exocytosis.

\textbf{Materials and Methods}

\textbf{Cell culture}

All chemicals used in our experiments were obtained from Sigma (St. Louis, MO) and all cell culture solutions were purchased from Invitrogen (Carlsbad, CA), or Sigma (St. Louis, MO) unless otherwise stated. Animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. Cultures of purified murine astrocytes and mixed cultures of astrocytes and neurons were generated as described previously (2). DF-1 cells, a spontaneously immortalized chicken
cell line (ATCC cat # CRL-12203) were propagated as a continuous line in MMEM.

**Freshly-isolated cells**

Cells were freshly isolated from mouse hippocampus using methods modified from those delineated in a previous publication (20). One-week old pGFAP-GFP mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with halothane and decapitated. The hippocampi were quickly dissected out and cut into small pieces, treated with EBSS (5%CO₂/95%O₂) containing 1mg/ml protease IV (Sigma, St. Louis, MO) at 37°C for 30 minutes. Then they were rinsed with artificial cerebrospinal fluid (aCSF, in mM: NaCl, 120; KCl, 3.1; NaH₂PO₄·H₂O, 1.25; CaCl₂·2H₂O, 2; MgSO₄·7H₂O, 1; NaHCO₃, 25; D-glucose, 10) and after 15-minutes of recovery were sequentially passed through glass Pasteur pipettes, dissociated and immediately deposited onto poly-L-lysine-coated coverslips. Cells were either fixed directly for immunocytochemistry or transferred to a chamber for visualization. Pipettes prepared from borosilicate capillaries (OD: 1.5mm, World Precision Instruments, Sarasota, FL) were used to aspirate individual identified astrocytes and neurons for RT-PCR. 10μL RNase-free solution, containing 1-2 U/μL RNasin (Promega, Madison, WI), 0.002 U/μL RQ1 RNase-free DNase (Promega) and 1mM DTT, was introduced into the pipettes. The pipettes were mounted on an Eppendorf 5171 Micromanipulator (Eppendorf AG, Hamburg, Germany). After a cell was identified based both on GFP fluorescence and morphology, a pipette was slowly positioned close to the cell and suction applied manually. The pipette was immediately transferred to a 0.2 ml Eppendorf tube and its tip broken to allow the contents to enter the tube. The tube was immediately put into an ethyl alcohol/dry ice bath to be frozen. For blank control, a small volume of saline adjacent to cells was collected in the same manner. To control for DNA contamination, 1-2 U/μL RNasin was replaced by 0.1-0.2 U/μL RNase ONE (Promega).
Reverse transcription PCR

Total RNA was extracted from brains of 1-week-old Swiss Webster mice (Charles River Laboratory, Wilmington, MA) or 7- to 10-day old pure astrocyte cultures using an RNeasy Mini kit (QIAGEN, Valencia, CA). For each experiment, 100 pg of total RNA was used. For single cell RT-PCR, aspirated cells were first incubated at 94°C for 10 min and reagents then added. The primers used in RT-PCR or PCR are listed in supplemental table 1. Superscript One-step RT-PCR with Platinum Taq (Invitrogen) was used for the first step RT-PCR and Platinum PCR Supermix (Invitrogen, Carlsbad, CA) for the second step PCR. All reactions were performed in a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA). All RT-PCR and PCR products were examined using conventional DNA electrophoresis and images taken using a custom imaging system. PCR products were found to have the predicted size based on the target sequence. Products were sequenced to confirm identity.

Western detection

Total proteins were extracted from brains of 1-week-old Swiss Webster mice or 7- to 10-day old pure astrocyte cultures. Tissue or cells were resuspended in RIPA buffer (50mM Tris base, 10mM NaCl, 1% NP-40, 5mM EDTA, 0.5% Na-Deoxycholate, 0.1% SDS, 2% PMSF, 1mM DTT, 1% protease inhibitor cocktail and pH 8.0) and the solution passed through a 27-gauge needle 10 times. The preparation was centrifuged at 10,000×g at 4°C for 1 min. The supernatant was aliquoted and stored at -80°C after total protein concentration was determined by O.D. meter. 2 µg of protein was loaded into each lane from both astrocyte cultures and whole brain. Western detection was performed as described (13).

Immunocytochemistry and immunofluorescence microscopy

Immunocytochemistry was performed as described previously (17). Antibodies and the dilutions used in
these studies can be found in supplemental table 2. Confocal images were obtained using a three-channel confocal microscope (Prairie Technologies, Middleton, WI). The lack of bleedthrough between different channels was confirmed by performing single-labeled control samples at the same laser intensity and PMT settings as used for triple-labeled samples. Stacks of images were processed and reconstructed using Metamorph software (Universal Imaging, West Chester, PA). Anti-GFAP was purchased from Sigma Chemical Company (St. Louis), Anti-Munc18, SNAP-23, synaptobrevin II, VGLUT1, complexin I/II, and synaptophysin purchased from Synaptic Systems (Goettingen, Germany) and ant-VGLUT2 was a gift from Dr. Robert Edwards (UCSF). In all experiments controls were performed in which primary antibodies were omitted to test for non-specific reaction of secondary antibodies. Western blots confirmed that each antibody reacted with a single band of the expected molecular weight.

**Virus preparation and application**

In order to get efficient and persistent gene expression selectively in astrocytes a strain of transgenic mouse, *Grv-a*, was used along with a retrovirus vector, pRV9, for astrocyte-specific gene transfer (21). These transgenic mice express TVA, a cell surface receptor for avian leukosis virus subgroup A (ALV-A) under the control of the astrocyte-specific promoter pGFAP. Because TVA is necessary for ALV-A to enter cells, astrocyte-specific expression of TVA renders glial cells susceptible to infection only with ALV-A vectors, such as pRV9. After DF-1 cells, reached 70%-80% confluence, they were transfected with pRV9 constructs by calcium phosphate-mediated transfection and kept in flasks for 4-6 days for sufficient infection of ALV-As. DF-1 cells were then enzymatically dissociated and stored in liquid N2 in MMEM containing 12% DMSO. Frozen DF-1 cells were replated in flasks and cultured to confluence, then 5 ml of fresh medium (MMEM for purified cortical astrocyte cultures, mito-MEM for primary astrocyte-neuron co-culture) was added into the flasks for 24 hr to be infected with virus
particles. The medium was subsequently centrifuged at 3,400×g to sediment the cell debris. The virus-containing medium was stored at 4°C prior to being used within one week. Application of ALV-As was performed as described (21). Briefly, virus-containing medium was applied 12 hr after the dissociated cells from 0-day Gtv-a mice were plated on the coverslips or flasks.

**Constructs**

To make the pRV9/synaptobrevin II (SNARE)-IRES-EGFP construct, DNA for synaptobrevin II residues M1-M96 was amplified from pCDM8/synaptobrevin II by 5’-end primer (5’-AATACGACTCACTATAGGGAGAC-3’) and 3’-end primer (5’-GGAATTCTACATCATCTTGAGGAGGCC-3’). Meanwhile, EGFP mRNA was amplified from pEGFP (Clontech, Palo Alto, CA) by 5’-end primer (5’-TCGACTCTAGAGGATCCCGGTACC-3’) and 3’-end primer (5’-TGGAAATTCTAGAGTCCGACGGCCGCTTTACT-3’). In an adapter vector, pBSFI/IRES, EGFP fragment was inserted into 3’-end of IRES (between BamHI and SalI sites), and synaptobrevin II (SNARE) fragment was inserted into 5’-end of IRES (at EcoRI site). The fragment of synaptobrevin II (SNARE)-IRES-EGFP was then subcloned into pRV9 by SfiI.

**Electrophysiology**

In neuron-astrocyte co-cultures, mechanical stimulation of astrocytes leads to a glutamate-dependent slow inward current (SIC) in adjacent neurons (2). This current was used as one monitor of glutamate release from astrocytes after the expression of EGFP and the synaptobrevin II SNARE domain. Whole-cell patch-clamp recordings were obtained as described previously (2). The morphological identification of neurons was confirmed electrophysiologically by their ability to generate TTX-sensitive Na+-mediated action potentials and by the presence of fast synaptic currents. Astrocytes were stimulated
mechanically using glass micropipettes filled with external control solution. Pipettes were slowly manipulated onto the surface of an astrocyte using a motorized MP285 micromanipulator (Sutter Instruments; Novata, CA) until a change in internal calcium was stimulated as monitored by a calcium indicator. During the application of a stimulus the user monitored the calcium signal to provide feedback concerning the timing of contact with the cell surface. On observation of an evoked calcium response the pipette was retracted. The use of this approach prevents damage to astrocytes which we independently confirmed by the absence of dye leakage from stimulated cells. For each neuron, up to four different astrocytes were stimulated at intervals of >1 min. Unless otherwise stated, at least eight astrocytes were stimulated in each parallel control and test condition, and data were obtained from at least three different cultures. The incidence of astrocyte-induced responses was defined as the proportion of responses relative to the total number of astrocytes stimulated in each experiment. Therefore, for these variables, \( n \) corresponds to the number of preparations, whereas for the other variables, such as the amplitude of the SIC, \( n \) represents the number of cells examined. Statistical differences were established using the Student’s \( t \)-test, unless otherwise stated. All experiments were performed at room temperature (20-23°C).

**Glutamate measurement**

An enzymatic assay for extracellular glutamate was used to visualize glutamate release from cultured astrocytes (22). In the presence of glutamate released from astrocytes, L-glutamic dehydrogenase (GDH) reduces \( \beta \)-nicotinamide adenine dinucleotide (NAD\(^+\)) to NADH, a product that fluoresces when excited by UV light. Coverslips containing purified astrocytes were mounted in a perfusion chamber and bathed in GDH saline, which consisted of saline supplemented with GDH (~56 U/ml) and NAD\(^+\) (1 mM). NADH fluorescence was excited by a xenon arc lamp (100 W) using a D360/10X (Chroma Technology...
Corp., Brattleboro, VT) exciter filter, 510DRLP dichroic mirror (Omega Optical, Brattleboro, VT) and 515EFLP emission filter (Omega Optical). Background subtraction of the fluorescent signals was performed by subtracting values recorded from the cells bathed in the solution lacking GDH and NAD⁺. Data were expressed as $\frac{F}{F_0} (%)$, where $F_0$ represents the fluorescence level of the optical field before stimulation and $F$ represents the change in fluorescence. When using this approach $F_0$ represents the sum of fluorescence emitted from GDH plus NAD⁺ plus basal NADH (either as a contaminant or because of enzymatic activity). For each different experimental condition, data were collected from at least three different cultures. Statistical significance was established using the ANOVA followed by student’s $t$-test.

It should be noted that in comparison to a previous study (22) in which we used this glutamate assay we detect smaller changes in NADH fluorescence. This reduction in signal is because in the current study we use perfusion in our chamber, while in the former we used a static bath.

**Intracellular Ca²⁺ measurement**

The elevation of $[\text{Ca}^{2+}]_i$ in astrocytes was monitored using the Ca²⁺ indicator X-rhod-1. Cultures were loaded with X-rhod-1, AM (5 µg/ml) for 30 minutes at 37°C. All changes in fluorescence are reported as background-subtracted $\frac{F}{F_0}$.

**Immunoelectron microscopy**

All procedures were approved by the Institutional Animal Care and Use Committee at Jefferson Medical College of Thomas Jefferson University and conformed to National Institutes of Health guidelines. Six
adult male Sprague-Dawley rats (Harlan, Indianapolis, IN; 200–250 g) were used for the immunoelectron microscopy study. Animals were deeply anesthetized with sodium pentobarbital and perfused transcardially through the ascending aorta with 50 ml of 3.8% acrolein (Electron Microscopy Sciences, Fort Washington, PA) and 200 ml of 2% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.4). Immediately following perfusion–fixation the brains were removed, cut into 13 mm coronal slices and placed in the same fixative for an additional 30 min. 40 μm thick sections were cut with a vibratome through the hippocampal formation, placed for 30 min in 1% sodium borohydride in 0.1 M PB to remove reactive aldehydes and rinsed extensively in 0.1 M PB before the primary antibody incubation. Methods for dual immunocytochemical labeling have been described previously (23). Tissue sections containing the hippocampus were incubated at room temperature for 15-18 hr in a cocktail containing a rabbit polyclonal anti-VGLUT1 antibody (1:400; Synaptic Systems) and a mouse monoclonal anti-S-100β (1:400; Sigma) in 0.1M tris buffered saline (TBS; pH 7.6). Sections were rinsed three times in 0.1M TBS and incubated in the secondary antiserum at room temperature. Visualization of VGLUT1 and S100β was conducted using two labeling techniques. In one series, S-100β was visualized using immunoperoxidase detection while VGLUT1 was detected using immunogold-silver labeling. For this combination, sections were incubated in biotinylated goat anti–mouse IgG (1:400, Vector Laboratories) for 30 min followed by a 30 min incubation in avidin–biotin complex (Vector Laboratories). Subsequently sections were placed in a goat anti–rabbit IgG conjugated to 1nm gold particles (Amersham Corp., Piscataway, NJ) for 2 hrs at room temperature followed by silver enhancement of the gold particles. In another series, VGLUT1 was detected using immunoperoxidase labeling where the secondary antibody was a biotinylated goat anti–rabbit IgG (1:400, Vector Laboratories). Following a 30 min incubation in the secondary antibody and a 30 min incubation in avidin–biotin complex (Vector Laboratories), VGLUT1 was visualized using a 4 min reaction in 22 mg of 3–3’ dianinobenzidine and 10
μl of 30% hydrogen peroxide in 100 ml of 0.1M TBS. For S-100β immunogold-silver labeling, sections were subsequently rinsed in 0.01M phosphate-buffered saline (PBS) and incubated in a solution of 0.01M PBS containing 0.1% gelatin and 0.8% bovine serum albumin (BSA) for 30 min. Sections were then incubated in a goat anti–mouse IgG conjugated to 1nm gold particles (Amersham Corp., Piscataway, NJ) for 2 hrs at room temperature. These were rinsed in 0.01M PBS containing the same concentrations of gelatin and BSA as described above and subsequently rinsed with 0.01M PBS alone. Sections were then incubated in 1.25% glutaraldehyde in 0.01M PBS for 10 min followed by a wash in 0.01M PBS and then in 0.2M sodium citrate buffer (pH 7.4). Silver intensification of gold particles was achieved using a silver enhancement kit (Amersham Corp.). The optimal silver enhancement times were determined empirically for each experiment and averaged 12-13 min. For electron microscopy, sections were rinsed in 0.1M PBS and incubated in 2% osmium tetroxide in 0.1M PB for 1hr, washed in 0.1M PB, dehydrated and flat-embedded in Epon 812. Thin sections of approximately 80-100 nm were cut from the outer surface of the tissue with a diamond knife (Diatome) using a Leica ultramicrotome. These were collected on grids and counterstained with uranyl acetate and Reynolds lead citrate. Captured images of selected sections were compared with captured light microscopic images of the block face prior to sectioning.

Sections processed in the absence of primary antibodies did not exhibit immunoreactivity. To evaluate possible cross-reactivity of labeling of the primary antiserum by secondary antisera, some sections were processed for dual labeling with omission of one of the primary antisera. Tissue sections from animals with good preservation of ultrastructural morphology and with both markers clearly apparent were used for the analysis. At least 10 grids containing 5-10 thin sections each were collected from at least two plastic-embedded sections from each animal. A profile containing a small number of gold-particles (e.g. two gold particles) that was unlabeled in adjacent thin sections was designated as lacking detectable
immunoreactivity. As observed in low magnification electron micrographs, background labeling in the neuropil, deemed spurious, was not common; therefore, cellular compartments containing at least three gold particles were considered to be immunoreactive.

The frequency of VGLUT1 immunoreactivity in S100β-labeled profiles was determined by random sampling of portions of the hippocampal neuropil that contained both immunolabels. This was done to ensure that labeling differences did not result from uneven penetration of antibodies in thick tissue sections. Counting the same profile in adjacent ultrathin sections was avoided by analyzing sections that were non-overlapping and separated by at least 400 nm. Due to known differences in the sensitivity and resolution of immunogold and immunoperoxidase markers, sections were processed with reversal of the labels for VGLUT1 and S100β. As semi-quantitative data obtained were independent of the labeling technique used, data was pooled. The number of VGLUT1-labeled profiles was tallied with respect to localization in axons or axon terminals or glial cells. Data is represented as percentage of total labeled profiles analyzed.

**Membrane capacitance measurements**

Compensated membrane capacitance (C_m) measurements were made using a SWAM IIB patch clamp/lock-in amplifier (Celica, Ljubljana, Slovenia) operating at 800 Hz lock-in frequency (24). Upon establishment of the whole-cell configuration, C_m and G_a (access conductance) were compensated by C_slow and G_a compensation controls. A sine voltage of 111 mV (rms) was applied. The phase angle setting was determined both by a 1 pF calibration pulse and by monitoring the projection of this pulse from the C (signal proportional to C_m) to the G output of the lock-in amplifier (asterisks in Fig. 7).
Simultaneously, we recorded filtered (300 Hz, 4 pole Bessel) C and G signals and the NADH fluorescence intensity from a C660 photon counter (Thorn EMI, U.K.). The PhoCal program (LSR, UK) was used to acquire signals every 5 ms. Fluorescence and capacitance signals were digitally filtered at 0.1 and 5 Hz respectively (2-way 100th and 15th order FIR filter, Math Works MATLAB) and re-sampled at 20 Hz. To correct for slow drift in measurements of whole cell capacitance that occurred in some cells, we used a linear regression analysis to subtract the drift seen prior to application of trans-ACPD (100 μM). The pipette solution contains 140mM potassium gluconate, 10mM KCl, 2mM MgCl₂, 10mM KOH/HEPES and 2mM Na₂ATP, pH=7.35. Cells were bathed in GDH saline. All recordings were made at room temperature. Cells were voltage clamped at a holding potential of 50 mV. The average cell capacitance was 15.7 ± 1.3 pF (n=55; mean ± S.E.M). Recordings were made with pipette resistances between 1 and 4 MΩ (measured in pipette solution), giving access conductance of more than 80 nS. Trans-ACPD was prepared as stock solution (dissolved in H₂O [20 mM], diluted 1:4 in bath solution [pH=7.35]) and added as a bolus into the bath solution for stimulation giving the final concentration of 100 μM.

Results

As a first step towards testing a vesicle-mediated exocytosis mechanism of glutamate release we determined whether astrocytes express exocytotic proteins. Using reverse transcription polymerase chain reaction (RT-PCR) we identified the presence of transcripts coding for several vesicle proteins in astrocytes (Fig. 1A). It is well established that the formation of a macromolecular SNARE complex is essential for exocytosis (25). We confirmed the results of previous culture studies as well as other studies
of non-neuronal cells (26-29) by demonstrating the presence of transcripts for the SNARE proteins, synaptobrevin II and SNAP-23 (13;15;30), in astrocytes and additionally demonstrated that munc-18-1 and complexin 2, proteins that associate with SNARE proteins but do not comprise part of the core complex, are present in primary astrocyte cultures (Fig. 1). Western detection (Fig. 1B) and immunocytochemistry (Fig. 1C and data not shown) confirmed the presence of these proteins in primary astrocytes. Although these particular proteins are present in both astrocytes and in neuronal presynaptic terminals, astrocytes do not express all of the proteins present in neuronal synaptic terminals; for example, synaptophysin (Fig. 1), a neuronal synaptic protein, is not present in cultured astrocytes. If exocytosis mediates the release of glutamate astrocytes must express the vesicular glutamate transporters required to fill the vesicle with this chemical transmitter. RT-PCR, Western detection and immunocytochemistry demonstrated the presence of vesicular glutamate transporters 1 and 2 (VGLUT1, 2) (31;32) in primary astrocytes (Fig. 1).

A concern with studies of cultured astrocytes is whether such studies accurately reflect physiological processes in vivo; thus to begin to corroborate our studies in culture we used astrocytes freshly isolated from the hippocampus (33). After enzymatic treatment and trituration it is possible to isolate individual astrocytes from the hippocampus such that they maintain their fine ramified in vivo structure. Figure 2A shows a typical astrocyte freshly isolated from the hippocampus of a transgenic mouse in which the astrocyte-specific glial fibrillary acidic protein (GFAP) promoter drives the expression of GFP (34). Individual isolated astrocytes, or in some cases neurons, were aspirated into a pipette and subjected to single-cell multiplex RT-PCR to determine whether they contain transcripts for the vesicle proteins identified in our primary culture experiments. Figure 2B demonstrates that GFAP-positive astrocytes do contain transcripts for the SNARE proteins synaptobrevin II and SNAP-23 as well as the SNARE
associated proteins munc-18-1 and complexin 2. Additionally, we detected transcripts for VGLUT1 and 2 (Table 1). Because it is known that neuronal dendrites contain many mRNAs, we were concerned that we might amplify transcripts from fragments of dendrites that could be isolated along with the astrocyte. To control for this possibility, we determined whether we could detect dendritically-localized mRNA for αCAM Kinase II (35). While this was reliably detected in neurons (Fig. 2B; Table 1) it was never detected in astrocytes, confirming that we selectively amplified only astrocytic transcripts when using these freshly-isolated cells.

Immunocytochemistry performed on freshly-isolated astrocytes revealed the presence of the proteins encoded by the transcripts for the exocytotic proteins studied. Figure 2C shows a representative example from an acutely-isolated GFP-expressing astrocyte on which we performed double-labeling for the neuronal synaptic protein synaptophysin and for VGLUT1. Because nerve terminals are intimately associated with the processes of astrocytes, we immunolabeled for synaptophysin, a vesicle protein that is not expressed by astrocytes, in order to disclose the location of synaptic terminals. This micrograph clearly demonstrates that, in addition to the astrocytic processes, pinched-off nerve terminals, as recognized by anti-synaptophysin immunoreactivity, remain attached to freshly-isolated astrocytes, while synaptophysin immunoreactivity is not detected within the processes of the astrocyte. Although VGLUT1 is expressed in some, but not all, nerve terminals, it was also found within GFP-positive astrocytic processes (Fig. 2C). Because of concern that this apparent VGLUT1 immunoreactivity within astrocytes may represent immunoreactivity within attached nerve terminals we also reconstructed these optical volumes, and viewed the immunostaining from a 90 degree rotation. The insets in Figure 2C clearly show that VGLUT1 immunoreactivity is localized within the volume of the GFP-positive astrocytic process confirming the presence of this protein within astrocytes. In addition to VGLUT1
(89% immunopositive astrocytes), we also confirmed the presence of synaptobrevin II, SNAP-23 (89% of astrocytes), complexin 1/2 (56% of astrocytes), munc-18 (80% of astrocytes), and VGLUT2 (40% of astrocytes) immunostaining within GFP-labeled astrocytic processes (Fig. 3; Table 1). These results demonstrate that both the transcripts and protein for a variety of vesicle proteins are present within acutely-isolated astrocytes.

**VGLUT1 expression in astrocytes in situ**

To ask whether vesicular glutamate transporter is present within astrocytes in situ we performed double-label immuno-electron microscopy on tissue sections taken from perfusion fixed rat brains. Initially astrocytes were labeled with anti-S100β that was visualized by an immunoperoxidase reaction and VGLUT1 was identified using silver-intensified gold particles. Figure 4 demonstrates the presence of VGLUT1 immunostaining in nerve terminals (VGLUT-t) as well as within the processes of astrocytes that were double labeled with anti-S100B. In these figures it should be noted that VGLUT1 immunostaining is present within astrocytic processes adjacent to synapses; for example, Fig. 4C shows an astrocytic process containing a silver-intensified gold particle adjacent to an unlabelled nerve terminal (ut) and dendritic processes (d). Immunoreactivity was quantified by determining the frequency of immunoreactivity. 51% (n = 2127) of axon terminals while 25% of astrocyte processes (n = 386) were immunopositive for VGLUT1 (four tissue sections through the hippocampus of two rats were quantified). Silver-intensified gold particles were not detected in the absence of primary antibody. To confirm that S100B labeled astrocytic processes we detected anti-S100β using silver-intensified gold particles and VGLUT1 with immunoperoxidase labeling. In agreement with these previous results we detected anti-VGLUT1-dependent peroxidase labeling within S100B positive processes in addition to labeling VGLUT1 positive nerve terminals. That these processes were indeed astrocytic was confirmed by the
presence of characteristic astrocytic filaments and by sites where gap junctions formed with a neighboring cell. Together, this immunostaining pattern demonstrates the presence of VGLUT1 within astrocytic processes in situ in locations adjacent to synaptic terminals.

**SNARE proteins and glutamate release**

Given that cultured astrocytes, freshly-isolated astrocytes and astrocytes in situ express the same exocytotic proteins we returned to cell culture to ask whether this exocytotic protein machinery is necessary for the release of glutamate from astrocytes. We used a fluorescent assay to detect glutamate released from astrocytes (22) and determined that the addition of a physiological level of ATP (20µM) causes a purinergic receptor-dependent increase in internal calcium, which in turn stimulates the release of glutamate (Fig. 5A). This release of glutamate is calcium-dependent because it is blocked by the calcium chelator BAPTA and the IP3 receptor antagonist 2-aminoethoxydiphenyl borate (2-APB (36)) (Fig. 5B,C). The calcium-dependent release of glutamate is reduced by the V-ATPase inhibitor bafilomycin A1, consistent with vesicular storage of released glutamate (17).

The formation of the protein complex termed the SNARE complex is known to be essential for the fusion of vesicles with the plasma membrane. Previous studies using clostridial toxins have implicated a SNARE complex as critical for the calcium-dependent release of glutamate from astrocytes (17;18;37). In these experiments, the toxin cleaves one protein of the SNARE complex, and as a consequence the full SNARE complex does not form. Because it is difficult to prove the existence of an exocytosis-mediated pathway of transmitter release unless the system is perturbed using many approaches, we have used an alternative tool to disrupt SNARE complex formation and asked whether it similarly blocks glutamate release. Previous studies have shown that the expression of an exogenous SNARE domain is sufficient to prevent
the formation of the endogenous SNARE complex, thereby preventing vesicle-mediated exocytosis of neurotransmitters (38-40). If the calcium-dependent release of chemical transmitter from astrocytes is indeed mediated by exocytosis, it follows that the expression of the synaptobrevin II SNARE motif should block the release of glutamate without affecting calcium signaling within astrocytes. To obtain efficient and persistent expression of the SNARE motif selectively in astrocytes we used a strain of transgenic mouse, Gtv-a, along with a retrovirus vector, pRV9, for astrocyte-specific gene transfer (21). These transgenic mice express TVA, a cell surface receptor for avian leukemia virus subgroup A (ALV-A), under the control of the astrocyte-specific promoter pGFAP. Because TVA is required for ALV-A to enter cells, astrocyte-specific expression of TVA renders glial cells susceptible to infection only with ALV-A vectors, such as pRV9. Using this retroviral system, we obtained reliable expression selectively within astrocytes. In control experiments we found that all cells that expressed EGFP (n = 167, 4 experiments) due to this retroviral system were GFAP-immunopositive and exhibited the characteristic structure of astrocytes in cell culture. Neurons were never infected. In our RT-PCR experiments it should be noted that we did detect GFAP transcript in one isolated neuron (Table 1). However, because RT-PCR is so sensitive, it is likely that this represents a false-positive rather than the presence of GFAP within neurons. The expression of the SNARE domain did not affect the viability of astrocytes for at least two weeks in culture. Astrocytes maintained physiological processes such as calcium signaling and were able to proliferate after SNARE domain expression.

To ask whether the expression of the SNARE motif blocked calcium-dependent release of glutamate we performed two independent assays. First, we used the glutamate dehydrogenase-based enzymatic assay for glutamate release and determined that purinergic receptor-induced glutamate release is blocked by the expression of the SNARE motif (Fig. 5B). Second, we used co-cultured neurons as assays for released
glutamate and demonstrated that the glutamate-dependent slow-inward current induced in co-cultured neurons by mechanical stimulation of astrocytes (2) was reduced in incidence and amplitude by the expression of the SNARE motif (Fig. 6A, B). It should be noted that the reduced suppression of glutamate release by SNARE domain expression in this experiment compared to that shown in figure 5 results from a lower expression efficiency (74.6 ± 3.5%) in neuron-astrocyte co-cultures compared to pure astrocyte cultures (>99%) that results from the need to inhibit cell division in co-cultures. While, the expression of the SNARE motif and EGFP reduced the release of glutamate in each of these assays, the expression of EGFP alone had no effect (Figs. 5B, 6A,B). Furthermore, the inhibitory effect of the SNARE motif was at a site downstream of the calcium signal because its expression neither reduced the ATP nor the mechanical stimulus (not shown) induced calcium signal.

A critical test of a vesicle-mediated mechanism of transmitter release is to determine whether the stimulus that induces transmitter release causes the addition of vesicle membrane to the plasma membrane. To achieve this goal we made whole-cell capacitance measurements to monitor the surface area of the plasma membrane (41). Exocytosis and the resulting increase in membrane surface area is detected as an increase in whole-cell capacitance. We have recently demonstrated that flash photolysis of the calcium cage NP-EGTA evoked a step increase in internal calcium which in turn stimulated the tetanus toxin-sensitive exocytotic fusion of vesicular membrane with the plasma membrane as assayed by an increase in whole-cell capacitance (55). Because calcium-dependent exocytosis is detectable in astrocytes, we asked whether physiological activation of metabotropic receptors by trans-ACPD (100 µM) that induces the calcium-dependent release of glutamate (18) caused a correlated increase in membrane capacitance and release of glutamate. This is an experimentally challenging experiment because it requires that the whole cell recording does not washout the machinery required to couple receptor
activation to calcium signaling and exocytosis (42). To overcome problems of washout, we used as our
criterion for accepting a cell in our analysis that the end-point, glutamate release was detected. Then we
asked whether there was a correlated change in membrane capacitance. Application of trans-ACPD (100
µM), evoked a detectable increase in extracellular NADH fluorescence in eight cells. In five of these cells,
we detected a significant increase in whole-cell capacitance (2.4 ± 0.8 pF, n =5; Fig. 7). In contrast,
addition of a saline control neither evoked a change in capacitance (0.0 ± 0.1 pF, n = 3) nor glutamate
release. Because small “synaptic” vesicles are estimated to contribute 50-100 aF capacitance on fusing
with the cell membrane (41), and our noise levels can be limited to around 50 fF, it is not surprising that
we did not detect individual fusion events. Although single fusion events can be detected, their detection
either needs cell-attached recordings (43;44) in which case we would not have the sensitivity to
simultaneously monitor glutamate release, or requires the averaging of tens of thousands of events (45)
which is not feasible in this study.

Discussion

This study has provided a sequence of novel insights into the mechanism of calcium-dependent glutamate
release from astrocytes. We demonstrate an array of vesicle proteins in astrocytes freshly isolated from
the nervous system including the SNARE proteins synaptobrevin II, SNAP-23, SNARE associated
proteins Munc18 and complexin 1 / 2 as well as VGLUT1 and VGLUT2. The presence of the SNARE
proteins is not surprising given that non-neuronal cells are known to express synaptobrevin II and
SNAP-23 (26-29). However, we go on to demonstrate that the SNARE complex is essential for the
calcium-dependent release of chemical transmitter from astrocytes. Second, we demonstrate the
expression of VGLUT1 in astrocytic processes adjacent to synapses in situ which suggests that this
exocytic machinery is positioned so that as a consequence of releasing glutamate it could regulate
synaptic transmission. Finally, we demonstrate that the activation of metabotropic glutamate receptors to evoke a calcium elevation causes an increase in cell surface area which we interpret as being due to the simultaneous fusion of vesicles with the plasma membrane (rather than to an unfolding of the plasma membrane (46)) and the release of glutamate. Taken together these data lead us to suggest that glutamate is released from astrocytes by an exocytotic mechanism. However, it is still necessary to perform additional studies in which the presence of glutamate within astrocytic vesicles is identified. Coupled with other evidence, including sensitivity to tetanus and botulinum neurotoxins (17;18) and the presence of quantal release of glutamate (16), it is likely that astrocytes can release glutamate in an exocytotic manner. This conclusion seems at odds with the often-reported role for astrocytes in taking up presynaptically-released glutamate by plasma membrane transporters where it is then converted to glutamine within the astrocyte and shuttled back to neurons as a renewable source of glutamate.

Although cytosolic glutamate levels are consequently reduced in comparison to neuronal levels, astrocytes do contain cytosolic glutamate at low millimolar levels (47-49), a quantity appropriate for the operation of vesicular glutamate transporters which have $K_M$ values of 1-2 mM (31;50;51). Moreover, a recent electrophysiology study performed at the calyx of Held synapse in the auditory brainstem demonstrated that 1mM intra-terminal glutamate is sufficient to support stable glutamatergic synaptic transmission (52). When 1mM glutamate was dialyzed presynaptically, miniature synaptic currents were ~95% of the amplitude of synaptic currents in unperturbed preparations. Thus, even though astrocytes have a lower cytosolic glutamate content than neurons these low millimolar levels will be sufficient, based on VGLUT $K_m$ values and electrophysiological studies, to support the filling of glial vesicles with glutamate.

One concern about studies employing antibodies relates to their specificity of reactivity. However,
western blots confirmed specificity of reactivity, immunostaining required the presence of the primary antibody and our RT-PCR results confirmed that immunopositive cells did contain transcript coding for the proteins. Together, these data strongly support the notion that astrocytes do express VGLUTs, and that these proteins are present in astrocytic processes adjacent to synapses. A second concern with immuno-electron microscopy is whether reaction products diffuse away from the site of deposition and might therefore be located in astrocytic processes in error. The advantage of the immunogold detection method is that it provides a reaction product that does not diffuse substantially from the antigenic site (53-55).

However, a potential concern with silver enhancement of colloidal gold particles is self-nucleation that can lead to spurious silver precipitates in the tissue. The silver enhancement kit utilized in the present study is characterized by markedly delayed self-nucleation resulting in virtually no background deposits. As the process of silver enhancement of colloidal gold is time and temperature dependent, carefully controlled conditions were followed to prevent spurious gold-silver deposits. Isolated deposits of gold-silver particles that did not correspond to identified glial structures were negligible. In further support of the spatial specificity of reaction product, when silver-enhanced gold was used to localize S100 we did not find reaction product in nerve terminals.

When we performed measurements of whole cell capacitance while monitoring glutamate release through the accumulation of extracellular NADH we successfully detected the simultaneous insertion of new membrane and the release of transmitter in five of eight cells following stimulation by trans-ACPD. This provides compelling evidence to support an exocytotic mechanism of glutamate release. It is not clear why in three of these cells we failed to detect a change in whole cell capacitance. One possibility, however, is that the relative contribution of kiss-and-run transmitter release compared to full exocytosis varies between cells, as has been demonstrated in PC-12 cells expressing different ratios of synaptotagmin I and IV (56). Since kiss-and-run exocytosis will not be detected as an accumulating increase in membrane surface area, and because astrocytes express synaptotagmin IV (Zhang and
Haydon, unpublished observations), this would explain the ability to release glutamate without a corresponding change in cell surface area in this minority of the cells we studied. Though there are striking similarities between a synaptic vesicle release pathway and the pathway of glutamate release from astrocytes we cannot over emphasize that they are not the same. Though common proteins are expressed, astrocytes do not express the synaptic vesicle protein synaptophysin. Moreover, the speed of release from astrocytes is much slower than the release rate from nerve terminals. A comparison of the maximum rates of exocytosis shows that astrocytes are slower by at least two orders of magnitude than neuronal synapses (57).

When we detected changes in whole-cell capacitance following application of trans-ACPD they represented significant increases in cell surface area. For example, assuming a vesicle contributes 50-100aF capacitance on fusing with the cell membrane, the average increase in cell capacitance of 2.4 pF that we measured in cell culture corresponds to the fusion of 24,000-48,000 vesicles with the plasma membrane. Though this may seem a large number it is less striking when one considers that one astrocyte in situ makes contact with over 100,000 synapses in stratum radiatum (58). Therefore, it is not unreasonable to consider that an astrocyte can release glutamate through the fusion of tens of thousands of vesicles distributed across one hundred thousand synapses.

Our immuno-electron microscopy reveals that vesicular glutamate transporters are present in astrocytic processes adjacent to synapses. In addition to the importance of demonstrating spatial localization, this observation is also of importance because this protein was present in astrocytes in adult, unperturbed rodent brain. Thus, the expression of vesicle proteins in astrocytes is not a culture artifact, nor is it solely a property associated with young animals. What is not clear from this picture is how effective glutamate
released from astrocytes will be in activating extrasynaptic neuronal receptors in the face of the avid plasma membrane transporter systems. Brain slice studies have shown that the mobilization of calcium within astrocytes is able to cause the release of glutamate, as detected by a glutamate dehydrogenase assay (18). Given that this transmitter can be released and can escape the transporter systems, it is likely that released glutamate has actions at extrasynaptic locations remote from concentrations of reuptake transporters and so will have a longer lifetime in the extracellular space. Indeed, studies performed using MK-801 to cause an activity-dependent block of synaptic NMDA receptors have shown that glutamate released from astrocytes accesses extrasynaptic NMDA receptors to modulate synaptic transmission (7).

It is often asserted that astrocytes do not contain vesicles within their processes in situ. When one inspects micrographs where there are few spherical structures within an astrocytic process it is difficult to conclude that they represent vesicles, especially when compared with the hundreds of vesicles in adjacent nerve terminals. Given our conclusive demonstration that astrocytic processes in situ contain vesicle proteins a careful immuno-electron microscopy study is warranted. Certainly immunoelectron microscopy studies performed on cultured astrocytes have shown a large number of vesicles associated with the vesicle protein synaptobrevin II (15). Such an in situ study should determine the types of intracellular organelles associated with these proteins, as well as their frequency and location with respect to the synapse. Given the different signaling demands of a nerve terminal and an astrocyte it is perhaps not surprising that such a quantitative difference is present. The nerve terminal has to be capable of releasing transmitter in response to action potentials that can discharge at rates of up to 100 impulses/second. Since the time for recycling of vesicles in nerve terminals is calculated to be of the order of tens of seconds it is not surprising that the presynaptic terminal is filled with hundreds of vesicles. In contrast, the astrocyte, a slow signaling system in which calcium oscillations are at rates of
only up to four per minute, may not require such a density of transmitter-filled vesicles because vesicle recycling and reloading with transmitter is of a similar time domain to the calcium oscillations. Because there are few vesicles within the astrocytic processes in comparison to nerve terminals, it is not surprising that previous studies have failed to identify VGLUT1 immunoreactivity. However, one recent study in which immuno-electron microscopy was performed to disclose the location of VGLUT3 did identify immunoperoxidase labeling of a putative astrocytic process (50). It is therefore likely that astrocytes express all three forms of the vesicular glutamate transporter, VGLUT1, 2 and 3. Whether different astrocytes express different individuals, or whether a single astrocyte expresses combinations remains to be ascertained.

Having determined that exocytosis mediates the release of glutamate from astrocytes and that the mechanism for this release process is present in situ, it is necessary to re-evaluate the way in which we consider the synapse to be regulated. In addition to transmitters released by nerve terminals having the potential to modulate synaptic transmission, it is now appropriate to consider that transmitters released from synaptically-associated astrocytes at the tripartite synapse are also critically involved in regulating synaptic transmission. Since synaptically-released neurotransmitters regulate astrocytic calcium and since elevated astrocytic calcium causes the calcium-dependent exocytotic release of the gliotransmitter, glutamate, synapses and astrocytes are involved in an intercellular dialog whose result dictates the strength of the synapse. Because each astrocyte has the potential to integrate information from over 100,000 associated synapses (58), these glial cells could control both the cross-talk between synaptic terminals and the spatial synchronization of synaptic transmission.

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### Table 1. Transcripts detected in freshly-isolated astrocytes or neurons

| Gene               | Sample       | FIN (9) a | FIA (9) a | Bath (6) a | FIN with RNase (1) a | FIA with RNase (1) a | FIN w/ |
|--------------------|--------------|-----------|-----------|------------|---------------------|---------------------|--------|
| GAPDH              | 9            | 9         | 0         | 0          | 0                   | 0                   | 0      |
| GFAP               | 1            | 9         | 0         | 0          | 0                   | 0                   | 0      |
| CaMKII             | 9            | 0         | 0         | 0          | 0                   | 0                   | 0      |
| Synaptophysin      | 9            | 0         | 0         | 0          | 0                   | 0                   | 0      |
| Synaptobrevin II   | 9            | 9         | 0         | 0          | 0                   | 0                   | 0      |
| SNAP-23            | 9            | 9         | 0         | 0          | 0                   | 0                   | 0      |
| Munc-18            | 9            | 9         | 0         | 0          | 0                   | 0                   | 0      |
| Complexin 2        | 9            | 9         | 0         | 0          | 0                   | 0                   | 0      |
| VGLUT1             | 8            | 8         | 0         | 0          | 0                   | 0                   | 0      |
| VGLUT2             | 6            | 9         | 0         | 0          | 0                   | 0                   | 0      |

**a** Summary of the number of positive cells for each gene studied among freshly-isolated astrocyte (FIN), freshly-isolated neuron (FIN) and saline adjacent to cells (Bath) under different conditions.

**b** Summary of the fluorescence triple-label in FIA. A blind evaluation was carried out by three independent investigators. The percentage of FIA that express a given vesicle protein is reported. The number of cells examined is shown in parentheses. Note that these percentages represent the average numbers obtained by three independent investigators not involved in this study. These investigators were shown a representative example of a positive sample (the original image stacks from the images shown in figure 2C) as well as no-primary antibody controls and asked to score each test sample for positive immunoreactivity for the vesicle protein label that was present within the same voxel occupied by GFP-label but not by synaptophysin label. Typically, positive micrographs contained more than ten immunopositive puncta within GFP processes. In all cases X-Z and Y-Z projections confirmed the presence of immunostaining within the GFP-positive process.
Figure Legends

Figure 1  Machinery for regulated exocytotic glutamate release is present in cultured astrocytes.  A) Multiplex RT-PCR detects the presence of exocytotic protein transcripts in astrocyte cultures. B) Western detection demonstrates the presence of vesicle proteins in whole brain and in purified astrocytes (*).  C) Immunocytochemistry on purified astrocyte culture (GFAP) demonstrates the presence of vesicular glutamate transporter type I (VGLUT1) and type 2 (VGLUT2) immunoreactivity in a punctate distribution consistent with a vesicular localization of these proteins.  Astrocytes are immuno-negative for the neuronal synaptic protein, synaptophysin but positive for the SNARE protein synaptobrevin II.  A control micrograph is shown in which primary antibody was omitted from the immunofluorescence staining procedure.  Scale bar for C represents 50 µm and 10 µm for insert.

Figure 2  Machinery for regulated exocytotic glutamate release is present in astrocytes acutely isolated from the hippocampus.  A) Astrocytes were isolated from pGFAP-GFP transgenic mice and identified based on GFP fluorescence (top).  A phase-contrast micrograph of the same cell is shown (bottom).  Individual cells were collected with a micropipette for multiplex RT-PCR (scale bar, 20 µm).  B) Single astrocyte and single neuron multiplex RT-PCR show that freshly-isolated astrocytes contain transcripts for exocytotic machinery previously identified in cultured cells.  Note that neither synaptophysin nor the dendritic transcript αCAM Kinase II were amplified from single astrocytes.  C) Immunocytochemistry on freshly-isolated astrocytes from 1-week old pGFAP-GFP mice shows the presence of VGLUT1 within the processes of the astrocytes (Scale bar, 10 µm).  Insets in 2C show XZ and XY projections from the regions identified by the vertical and horizontal lines in part C, (Z calibration 2 µm).
Figure 3  Immunofluorescence-labeling shows the presence of vesicle-associated proteins in acutely isolated astrocytes. GFP-expressing hippocampal astrocytes were isolated from 1-week old pGFAP-GFP transgenic mice and double-labeled with two antibodies against a vesicle-associated protein and the presynaptic terminal specific protein, synaptophysin. Each row shows one example of a vesicle protein. The first image of each row shows the top-view maximal projection from a sequence of confocal images of one astrocyte (scale bar = 10 μm), and the white box indicates the region that is zoomed into in adjacent images. The next four images show the fluorescent signal due to GFP (blue), the presynaptic terminal marker synaptophysin (red), a vesicle protein (green), followed by a merged image. These images are obtained from two adjacent optical-sections separated by 0.5 μm. (scale bar = 2 ¼m). Red arrowhead represents anti-synaptophysin presynaptic terminal labeling; yellow arrowhead represents presynaptic terminals co-labelled with the vesicle-protein, while the turquoise arrowhead represents vesicle protein labelling located within the GFP positive processes of the astrocyte. Note that we detect immunolabelling within the astrocyte for each of the vesicle proteins shown.

Figure 4  Electron micrographs showing localization of the type I vesicular glutamate transporter (VGlut) using immunogold-silver labeling and S100 protein using immunoperoxidase labeling in the hippocampus.  

A) Differential localization of VGLUT1 in axon terminals (VGlut-t) and S100β protein in glial cells. Bar = 0.5 μm.  
B) A glial process exhibiting peroxidase labeling for S100 protein also contains immunogold-silver labeling for VGlut (arrowheads). An axon terminal containing gold-silver labeling for VGLUT1 can also be identified in the neuropil (VGlut-t). Bar = 0.4 μm.  
C) A glial process (VGlut + S100β) identified by peroxidase labeling for S100β that contains gold-silver particles indicative of VGLUT1 (arrowheads) is directly apposed by an unlabeled terminal (ut) and a dendritic process (d). Bar = 0.38 μm.  
D) Three axon terminals exhibit gold-silver labeling for VGLUT1 (VGlut-t). One
VGlut-t is apposed to a glial process (as demonstrated by S100 labeling) that exhibits gold-silver labeling for VGLUT1 (VGlut+S100). Bar = 0.45 µm.

**Figure 5** The release of glutamate from astrocytes is mediated by the formation of a SNARE complex. A) Purified astrocytes made from pGFAP-Tva transgenic mice were infected with avian leukosis virus type A (ALV-A) containing synaptobrevin II (SNARE)-IRES-EGFP or IRES-EGFP and assayed for the release of glutamate by detecting the accumulation of NADH in an enzymatic assay (see text). 20 µM ATP stimulates the release of glutamate (a), as detected by the accumulation of NADH. Almost no glutamate was released from astrocytes expressing synaptobrevin II (SNARE) in contrast to the astrocytes expressing only EGFP. B & C) PPADS (P2 receptor antagonist; 50 µM, 10 minute pre-incubation), BAPTA-AM (cell-permeable Ca²⁺ chelator loaded into cells for 1 hour at 10 µM) and 2-APB (IP₃-receptor antagonist; 150 µM, 10 minute preincubation) significantly decreased the ATP-induced [Ca²⁺]ᵢ elevation as well as glutamate release. Bafilomycin A1 (v-ATPase inhibitor; 5 µM, 90 minute pre-incubation) significantly reduced glutamate release with no effect on [Ca²⁺]ᵢ elevation. Expression of synaptobrevin II (SNARE) plus EGFP, but not EGFP alone, blocked glutamate release without affecting the ATP-induced calcium signal, a result consistent with exocytotic release of glutamate. * p<0.05, **p<0.01.

**Figure 6** Expression of synaptobrevin II SNARE domain significantly reduces membrane cycling and astrocyte-induced slow inward current in adjacent neurons. A) & B) Individual neuronal responses to astrocyte stimulation are seen as a glutamate-mediated slow inward current (SIC) which was reduced when astrocytes expressed the synaptobrevin II SNARE domain. The incidence (EGFP: 90.9±3.1%;
synaptobrevin II SNARE domain: 65.4±9.4%) and amplitude (EGFP: 117.0±19.5pA; synaptobrevin II
SNARE domain: 64.9±9.2pA) of the SIC were significantly decreased by expressing synaptobrevin II
SNARE domain (p<0.05 and p<0.01, respectively), but the magnitude of the Ca\(^{2+}\)-wave was unaffected
(data not shown).

**Figure 7** *Trans*-ACPD induces exocytotic release of glutamate. A) Simultaneous measurement of
 glutamate release and whole cell capacitance shows that the metabotropic glutamate receptor agonist
*trans*-ACPD stimulates exocytotic release of glutamate. Simultaneous recording of NADH fluorescence
(upper trace), cell capacitance (imaginary admittance signal, middle) and the real admittance signal
(bottom) contributed by access and membrane conductances, and by membrane capacitance in a cultured
astrocyte voltage clamped at a holding potential of –50 mV. Bar at the bottom indicates the application of
*trans*-ACPD (100 µM) into the bath solution. We detected a change in whole-cell capacitance in five of
eight astrocytes in which *trans*-ACPD evoked the release of glutamate. Asterisks denote calibration
pulses of 1 pF, used to set the phase of the lock-in amplifier. Note that the transient in the record is due to
changes in stray capacitance associated with application of *trans*-ACPD.
### Supplemental Table 1. RT-PCR and PCR primers

| Primer               | Sequence (5’3)                          | Tm (°C) | Product size (bp) | Reference sequence in GenBank |
|---------------------|-----------------------------------------|---------|-------------------|------------------------------|
| GAPDH-out5          | CCCACTAACATCAAATGGGG                   | 55      | 749               | NM_008084                    |
| GAPDH-out3          | ATGTAGGCCATGAGGTCAC                    |         |                   |                              |
| GAPDH-in5           | ATGTTTGTGATGGGTGTAACCAC                | 62      | 453               |                              |
| GAPDH-in3’          | CTGGTCCTCAGTGTAGCCCAAGAT               |         |                   |                              |
| GFAP-out5’          | ATGCCACGTTTCTCCTTGT                   | 55      | 632               | K01347                       |
| GFAP-out3’          | GCCTCGTATTTGATGGCAAT                  |         |                   |                              |
| GFAP-in5’           | GCCACGTTTCTCCTTGTCTCGA                | 62      | 435               |                              |
| GFAP-in3’           | GCTTCATGTGCCTCCTGTCTAT                |         |                   |                              |
| CaMKII-out5’        | CTGCTGAGAAGTGGGGAATC                  | 55      | 829               | NM_009792                    |
| CaMKII-out3’        | ACATTCACGGACAAAAGGAC                 |         |                   |                              |
| CaMKII-in5’         | CTGCTCCGACAGCTCCACTCTCTG             | 60      | 297               |                              |
| CaMKII-in3’         | AAGTCTCCATTGCTTGCTTATGCTTCCG         |         |                   |                              |
| Synaptophysin-out5’ | GGAGTGGGGTCATGTGACTT                 | 55      | 1000              | BC014823                     |
| Synaptophysin-out3’ | GGAAAGTGGGGGTTCTGGAG                 |         |                   |                              |
| Synaptophysin-in5’  | CCATTCTGTATGGGCGAGAAAG              | 60      | 400               |                              |
| Synaptophysin-in3’  | GATCAACAGCCTACCAACGTCAC             |         |                   |                              |
| SNAP-23-out5’       | AACCTGTCCTCCAGAGGAAAG           | 55      | 571               | AB000822                     |
| SNAP-23-out3’       | TGGTCAGCCTTTCTGTG                   |         |                   |                              |
| Gene          | Primer Pair           | Sequence               | Length (nt) | Length (bp) | Accession Number |
|--------------|-----------------------|------------------------|-------------|-------------|-----------------|
| SNAP-23-in5' | AGCCATTGAGTCTCAGGATGCAGGA | 62                     | 390         |             |                 |
| SNAP-23-in3' | CTGCCCACTTGGAGTCAGGTTCTC |                        |             |             |                 |
| Munc-18-out5' | ACTCGCTGACTCTTTCCAA | 55                     | 203         |             | XM_130124       |
| Munc-18-out3' | GTCGGCTTTTATAGGCATCCA |                        |             |             |                 |
| Munc-18-in5' | TCTACAGCCCCTCAACAAGCGG  | 60                     | 149         |             |                 |
| Munc-18-in3' | GATCAGCTGAGCCAGCAAGG |                        |             |             |                 |
| Synaptobrevin II-out5' | GTCACTG CCTCTGGCCAAGTC | 55                     | 409         |             | NM_009497       |
| Synaptobrevin II-out3' | GGCAGACTCCTCAGGGATTT |                        |             |             |                 |
| Synaptobrevin II-in5' | CTGCACCTCCTCCAACCTTAC | 60                     | 297         |             |                 |
| Synaptobrevin II-in3' | GGATTTAAGTGCTGAAGTAACGATG |                        |             |             |                 |
| Complexin 2-out5' | AAAGAGGCAGAGGAAGGC | 55                     | 320         |             | NM_009946       |
| Complexin 2-out3' | GGGAGGAGTCCTGACTAGG |                        |             |             |                 |
| Complexin 2-in5' | CAGGACATGTCTCAGAAGTAACCC | 60                     | 147         |             |                 |
| Complexin 2-in3' | GTAAGGGGGCAGAGGTCGACTA |                        |             |             |                 |
| VGLUT1-out5' | GTCTTGGGCTTGGCCATTGT | 55                     | 996         |             | XM_133432       |
| VGLUT1-out3' | CAGGGAGGCTATGAGGAACA |                        |             |             |                 |
| VGLUT1-in5' | CACTATGGCTGCTCATCTTCTG | 60                     | 617         |             |                 |
| VGLUT1-in3' | GTGGACATTATGTGACGACTGC |                        |             |             |                 |
| VGLUT2-out5' | GAAATCAGCAAGGGTGGGCAT | 55                     | 836         |             | AF324864        |
| VGLUT2-out3' | CAAGACCTTGGCTTTGATATGTT |                        |             |             |                 |
| VGLUT2-in5' | AGCAAGGGTGGCATGTTGTCTG | 60                     | 698         |             |                 |
| VGLUT2-in3' | CGGTCCCTTAGGTTACGCGT |   |   |   |   |
Supplemental Table 2. Antibodies and their working dilution

| Antibody       | Source                        | Type                        | Cat. No. | Dilution | Immunocytochemistry | Western blot |
|----------------|-------------------------------|-----------------------------|----------|----------|---------------------|--------------|
| GFAP           | Sigma, St Lious, MI           | Rabbit polyclonal, IgG      | G9269    | 1:80     | 1:200               |              |
| Munc18         | Synaptic Systems, Gottingen, Germany | Rabbit polyclonal, serum | 116002   | 1:1000   | 1:1000              |              |
| SNAP-23        | Synaptic Systems, Gottingen, Germany | Rabbit polyclonal, serum | 111202   | 1:50     | 1:1000              |              |
| Synaptobrevin II | Synaptic Systems, Gottingen, Germany | Mouse monoclonal, IgG1  | 104201   | 1:1000   | 1:10000             |              |
| Synaptophysin  | Synaptic Systems, Gottingen, Germany | Mouse monoclonal, IgG1 | 101001   | 1:1000   | 1:10000             |              |
| VGLUT1         | Synaptic Systems, Gottingen, Germany | Rabbit polyclonal, serum | 135002   | 1:1000   | 1:1000              |              |
| VGLUT2         | Dr. Robert H. Edwards, UCSF   | Rabbit polyclonal, serum    | NA       | 1:1000   | 1:1000              |              |
| Alexa Fluor 488 goat anti-rabbit IgG | Molecular Probes, Eugene, OR | Goat polyclonal, serum      | A-11008  | 1:1000   | NA                  |              |
| Alexa Fluor 546 goat anti-rabbit IgG | Molecular Probes, Eugene, OR | Goat polyclonal, serum      | A-11010  | 1:1000   | NA                  |              |
| Alexa Fluor 546 goat anti-mouse IgG | Molecular Probes, Eugene, OR | Goat polyclonal, serum      | A-11003  | 1:1000   | NA                  |              |
| Alexa Fluor 633 goat anti-mouse IgG | Molecular Probes, Eugene, OR | Goat polyclonal, serum      | A-21050  | 1:1000   | NA                  |              |
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