Journal of Neurochemistry, 2006, **96**, 656–668 doi:10.1111/j.1471-4159.2005.03631.x

Glia re-sealed particles freshly prepared from adult rat brain are competent for exocytotic release of glutamate

Sara Stigliani,* Simona Zappettini,* Luca Raiteri,* Mario Passalacqua,†,§ Edon Melloni,†,§ Consuelo Venturi,‡,¶ Carlo Tacchetti,‡,¶ Alberto Diaspro,** Cesare Usai†† and Giambattista Bonanno*,§

*Pharmacology and Toxicology Section, †Biochemistry Section and ‡Human Anatomy Section, Department of Experimental Medicine, University of Genoa, Genoa, Italy

§Center of Excellence for Biomedical Research, University of Genoa, Genoa, Italy

¶Italian Foundation for Cancer Research, Institute of Molecular Oncology (IFOM), Milan, Italy

**National Institute for the Physics of Matter (INFM) and Department of Physics, University of Genoa, Genoa, Italy

††Institute of Biophysics, National Research Council, Genoa, Italy

Abstract

Glial subcellular re-sealed particles (referred to as gliosomes here) were purified from rat cerebral cortex and investigated for their ability to release glutamate. Confocal microscopy showed that the glia-specific proteins glial fibrillary acidic protein (GFAP) and S-100, but not the neuronal proteins 95-kDa postsynaptic density protein (PSD-95), microtubule-associated protein 2 (MAP-2) and β-tubulin III, were enriched in purified gliosomes. Furthermore, gliosomes exhibited labelling neither for integrin-αM nor for myelin basic protein, which are specific for microglia and oligodendrocytes respectively. The Ca^{2+} ionophore ionomycin (0.1–5 μM) efficiently stimulated the release of tritium from gliosomes pre-labelled with [^{3}H]D-aspartate and of endogenous glutamate in a Ca^{2+}-dependent and bafilomycin A1-sensitive manner, suggesting the involvement of an exocytotic process. Accordingly, ionomycin was found to induce a Ca^{2+}-dependent increase in the vesicular fusion rate, when exocytosis was monitored with acridine orange. ATP stimulated [^{3}H]D-aspartate release in a concentration- (0.1–3 mM) and Ca^{2+}-dependent manner. The gliosomal fraction contained proteins of the exocytotic machinery [syntaxin-1, vesicular-associated membrane protein type 2 (VAMP-2), 23-kDa synaptosome-associated protein (SNAP-23) and 25-kDa synaptosome-associated protein (SNAP-25)] co-existing with GFAP immunoreactivity. Moreover, GFAP or VAMP-2 co-expressed with the vesicular glutamate transporter type 1. Consistent with ultrastructural analysis, several ~30-nm non-clustered vesicles were present in the gliosome cytoplasm. It is concluded that gliosomes purified from adult brain contain glutamate-accumulating vesicles and can release the amino acid by a process resembling neuronal exocytosis.

Keywords: exocytosis, gliosomes, glutamate release, SNARE proteins.

J. Neurochem. (2006) **96**, 656–668.

The role of glia in the brain is an area of intense investigation. In the past decade, exciting results in this field have led to dramatic conceptual changes about the role of glial cells, which were formerly thought to provide only structural and trophic support to neurones.

An increasing number of papers have suggested that glia share at least some of the features typical of neurones, particularly those concerned with excitatory neurotransmission (for a review see Haydon 2001). In fact, glial cells are

Received September 6, 2005; revised manuscript received September 13, 2005; accepted September 13, 2005.

Address correspondence and reprint requests to Dr Giambattista Bonanno, Department of Experimental Medicine, Pharmacology and Toxicology Section, Viale Cembrano 4, 16148 Genova, Italy.
E-mail: bonanno@pharmatox.unige.it

Abbreviations used: BAPTA-AM, 1,2-*bis*-(2-aminophenoxy)ethane-*N,N,N′*,N′-tetra-acetylmethylester; GFAP, glial fibrillary acidic protein; [^{3}H]D-ASP, [^{3}H]D-aspartate; LDH, lactate dehydrogenase; MAP-2, microtubule-associated protein 2; MBP, myelin basic protein; PBS, phosphate-buffered saline; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; PSD-95, 95-kDa postsynaptic density protein; SNAP-23/25, 23/25-kDa synaptosome-associated protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; VAMP-2, vesicular-associated membrane protein type 2; vGLUT-1, vesicular glutamate transporter type 1.

endowed with transporters that are able to remove glutamate from the extracellular space and this process is even more efficient than that effected by neurones (Mennerick and Zorumski 1994; Bergles and Jahr 1997). Owing to their intimate spatial relationship with neuronal synaptic contacts, astrocytes can respond directly to synaptically released messengers and, in turn, communicate via signalling substances with neurones. Several lines of evidence suggest that glutamate released from neurones can activate both ionotropic and metabotropic receptors located on astroglial cells, inducing an increase in intracellular Ca^{2+} (Dani *et al*. 1992; Porter and McCarthy 1996; Pasti *et al*. 2001), which is associated with glutamate release (Parpura and Haydon 2000; Pasti *et al*. 2001; Bezzi *et al*. 2004). Glutamate release can also be observed following other stimuli, including bradykinin (Parpura *et al*. 1994), prostaglandins (Bezzi *et al*. 1998), chemokines (Bezzi *et al*. 2001), 5-hydroxytryptamine (Meller *et al*. 2002) and ATP (Zhang *et al*. 2004b) receptor activation. The release of glutamate evoked by these agents is linked to Ca^{2+} delivery from intracellular stores.

The disclosure of this active role of glia has led to the model of the 'tripartite synapse' (Araque *et al*. 1999; Bezzi and Volterra 2001) in which, besides the important tasks carried out by presynaptic and postsynaptic neuronal elements, a pivotal role in regulating synaptic function, strength and plasticity is played by the glial cells surrounding the abovementioned structures.

Most studies of the release of glutamate from glia have been carried out using cultured astrocytes (for instance Parpura *et al*. 1994; Bezzi *et al*. 1998, 2001; Araque *et al*. 2000; Zhang *et al*. 2004a); few have exploited other systems such as astroglioma cells (Meller *et al*. 2002) or freshly isolated astrocytes (Rutledge and Kimelberg 1996). More recent studies have used brain tissue slices isolated from rat hippocampus, a model that also allows study of the neuronal responses to glutamate released from astrocytes and vice versa (Angulo *et al*. 2004; Fellin *et al*. 2005; Perea and Araque 2005). In the present study we used a preparation of glial subcellular particles (termed gliosomes) isolated from the brain of adult rat and purified essentially according to the method described by Nakamura *et al*. (1993, 1994). This preparation appears well suited to the investigation of the functional neurochemistry of mature astrocytes. In particular we show here that release of previously taken up [^{3}H]D-aspartate ([^{3}H]D-ASP) and of endogenous glutamate from gliosomes can be evoked by a mechanism resembling neuronal exocytosis.

Materials and methods

Materials

[^{3}H]D-ASP (specific activity 16.3 Ci/mmol) and horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit secondary antibodies were purchased from Amersham (Little Chalfont, UK). Acridine orange, ionomycin, bafilomycin A1, ATP, 1,2-*bis*-(2-aminophenoxy)ethane-*N,N,N′*,N′-tetra-acetylmethylester (BAPTA-AM), mouse anti-glial fibrillary acidic protein (GFAP), rabbit anti-GFAP and anti-β-tubulin III were obtained from Sigma Chemical Co. (St Louis, MO, USA). Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) was purchased from Alexis Italia (Vinci, Italy). Lactate dehydrogenase (LDH) antibody was obtained from Biotrend GmbH (Köln, Germany). Antibodies against 25-kDa synaptosome-associated protein (SNAP-25), 23-kDa synaptosome-associated protein (SNAP-23) and vesicular-associated membrane protein type 2 (VAMP-2) were purchased from Synaptic Systems GmbH (Gottingen, Germany) and anti-95-kDa postsynaptic density protein (PSD-95) from Affinity Bioreagents Inc. (Golden, CO, USA). Antibodies against S-100, microtubule-associated protein 2 (MAP-2), integrin αM, myelin basic protein (MBP), syntaxin, vesicular glutamate transporter type 1 (vGLUT-1) and goat anti-guinea pig fluorescein-conjugated secondary antibody were from Chemicon International (Temecula, CA, USA). Goat anti-rabbit Texas Red-conjugated and goat anti-mouse fluorescein-conjugated secondary antibodies were obtained from Calbiochem (La Jolla, CA, USA). Donkey anti-mouse Alexa Fluor 488-coniugated, donkey anti-rabbit Alexa Fluor 647-conjugated, donkey anti-rabbit Alexa Fluor 488-conjugated and goat anti-mouse Alexa Fluor 633-conjugated secondary antibodies were obtained from Molecular Probes Europe (Leiden, The Netherlands).

Animals

Adult male rats (Sprague–Dawley, weighing 200–250 g) were used. Animals were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light–dark schedule (lights on at 07.00 hours and off at 19.00 hours). Food and water were freely available. The experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results.

Preparation of gliosomes and synaptosomes

Animals were killed and the brain cortex quickly removed. Purified gliosomes and synaptosomes were prepared essentially as described by Nakamura *et al*. (1993, 1994). The tissue was homogenized in 10 volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris-HCl, using a glass–Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min, 1000 *g* at 4°C) to remove nuclei and debris, and the supernatant was gently stratified on a discontinuous Percoll® gradient (2, 6, 10 and 20% v/v in Tris-buffered sucrose) and centrifuged at 33 500 *g* for 5 min. The layers between 2 and 6% Percoll® (gliosomal fraction) and between 10 and 20% Percoll® (synaptosomal fraction) were collected, washed by centrifugation, and resuspended in physiological medium (140 mM NaCl, 3 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM HEPES, 10 mM glucose, pH 7.4). In the case of release experiments, the gliosomal and synaptosomal layers were washed and resuspended in the following physiological medium: 125 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 1 mM NaH_2PO_4, 22 mM $NaHCO_3$ and 10 mM glucose (aerated with 95% O_2 and 5% CO_2); pH 7.2–7.4. Gliosomal and synaptosomal proteins were measured according to Bradford (1976).

Immunofluorescence confocal microscopy

Gliosomes and synaptosomes (100 μg protein) were placed on to coverslips pretreated with poly-L-ornithine and maintained at 37°C for 30 min in a 5% CO_2 atmosphere to allow setting and sticking to the substrate. All the following procedures were conducted at room temperature (22°C). The preparations were fixed with 2% paraformaldehyde, washed with phosphate-buffered saline (PBS) and incubated for 5 min with 0.05% Triton X-100. After washing with PBS containing 0.5% serum albumin, the preparations were incubated 30 min with the primary antibodies diluted in PBS containing 3% albumin. The following antibodies were used: rabbit anti-LDH (1 : 20), mouse anti-GFAP (1 : 40), rabbit anti-GFAP (1 : 10), mouse anti-S-100 (1 : 10), mouse anti-PSD-95 (1 : 200), mouse anti-MAP-2 (1 : 50), mouse anti-β-tubulin III (1 : 300), mouse anti-integrin αM (clone OX-42; 1 : 25), mouse anti-MBP (clone RIP; 1 : 1000), mouse anti-VAMP-2 (1 : 500), anti-syntaxin-1 (1 : 1000), anti-SNAP-25 (1 : 500), anti-SNAP-23 (1 : 20) and guinea pig anti-vGLUT-1 (1 : 1000). After washing with PBS containing 0.5% serum albumin, the preparations were incubated for 30 min with the appropriate secondary fluorescein-, Texas Red-, Alexa Fluor 488-, Alexa Fluor 633- or Alexa Fluor 647-labelled antibodies in PBS containing 3% albumin and washed. Images were collected by confocal microscopy using a MRC1034 instrument (Bio-Rad, Hercules, CA, USA), equipped with 488 and 567 nm excitation lines, or by a three-channel TCS SP2 laser-scanning confocal microscope (Leica Wetzlar, Germany), equipped with 458, 476, 488, 514, 543 and 633 nm excitation lines. Images (512 × 512 × 8 bit) were taken through a Diaphot 200 (Nikon Tokyo, Japan) or a Leica DM IRE inverted microscope with a plan-apochromatic oil immersion objective 100 ×/numeric aperture 1.4. The light collection configuration was optimized according to the combination of chosen fluorochromes and sequential acquisition was performed to avoid cross-talk between colour channels. Spatial co-localization was analysed through two-dimensional correlation cytofluorograms accomplished by macro routines integrated as plug-ins in ImageJ 1.34f software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA). Red and green labels were considered as co-localized in the same pixel if their respective intensities (0–255, eight bit) were strictly higher than the threshold of their channels, determined by analysis of the colour histograms, and if the ratio of their intensities was strictly higher than 50%.

Electron microscopy

For ultrastructural analysis, purified gliosomes and synaptosomes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, post-fixed in 1% osmium tetroxide in cacodylate buffer 0.1 M, pH 7.2, *en bloc* stained with a 1% aqueous solution of uranyl acetate and dehydrated through a graded ethanol series. Samples were then embedded in LX112 (Polysciences Inc., Warrington, PA, USA), polymerized for 12 h at 42°C, followed by 48 h at 60°C. Grey-silver ultrathin sections were obtained using a Leica Ultracut E microtome, stained with uranyl acetate and lead citrate, and analysed with a FEI CM10 or Tecnai 12 G2 (FE1, Eindhoven, the Netherlands) electron microscope. To quantify the number of postsynaptic densities and mitochondria in gliosome or synaptosome fractions, 10 electron micrographs were obtained at 8900 × from ultrathin sections of each sample. Fields were selected in order to include the maximum number of structures on the micrograph. The number of postsynaptic densities and mitochondria were counted, and the area of the corresponding photographic field was measured.

Release experiments

Gliosomes and synaptosomes were incubated at 37°C for 15 min in the presence of 0.1 μM [^{3}H]D-ASP or without label (endogenous glutamate release experiments). In some experiments, synaptosomes were incubated for 30 min in the presence or absence of 0.5 μM bafilomycin A1 or 100 μM BAPTA-AM. At the end of the incubation period, aliquots of the gliosomal and synaptosomal suspensions were layered on microporous filters at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri *et al*. 1974; Raiteri and Raiteri 2000). Superfusion was started at a rate of 0.5 mL/min with standard medium aerated with 95% O_2 and 5% CO_2. After 36 min of superfusion, to equilibrate the system, four 3-min samples were collected. Stimulation with 0.1–5 μM ionomycin or 0.3–3 mM ATP was applied at $t = 39$ min. Ionomycin was administered as a 90-s pulse; ATP was present until the end of the experiment. PPADS was added to the superfusion medium at $t =$ 30 min. When used, the Ca^{2+}-free medium was introduced at $t =$ 20 min. Fractions collected and superfused gliosomes or synaptosomes were counted for radioactivity or analysed for their endogenous glutamate content. [^{3}H]D-ASP radioactivity was determined in each fraction collected and in the superfused filters by liquid scintillation counting. Endogenous glutamate was measured by HPLC analysis following pre-column derivatization with *o*-phthalaldehyde and separation on a C18 reverse-phase chromatographic column (10 × 4.6 mm, 3 μm; Chrompack, Middleburg, The Netherlands) at 30°C coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm; Raiteri *et al*. 2000). Buffers and the gradient programme were as follows: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol 80 : 20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20 : 80; solvent C, sodium acetate (pH 6.0)/methanol, 80 : 20; gradient programme, 100% C for 4 min from the initiation of the programme; to 90% A and 10% B in 1 min; to 42% A and 58% B for 14 min; to 100% B in 1 min; isocratic flow 2 min; to 100% C in 3 min; flow rate 0.9 mL/min. Homoserine was used as an internal standard. The radioactive neurotransmitter efflux in the samples collected during release experiments was calculated as fractional rate × 100. The endogenous amino acid release was expressed as nmoles per milligram of protein. The stimulus-evoked overflow of [^{3}H]D-ASP and endogenous glutamate was estimated by subtracting the appropriate basal release from the release evoked in the samples collected during and after stimulation.

Monitoring exocytosis

Vesicle membrane fusion was determined in gliosomes and synaptosomes using the fluorescent dye acridine orange as a probe (Zoccarato *et al*. 1999). Fluorescence was measured by a RF-5301PC (Shimadzu, Kyoto, Japan) spectrofluorophotometer at 490 and 530 nm excitation and emission wavelengths respectively. Aliquots (800 μg protein in 200 μL HEPES-buffered medium, either with or without Ca^{2+}) of the gliosomal or synaptosomal suspensions were stored on ice until use. Aliquots were diluted in HEPES-buffered physiological medium (final volume 2 mL) containing the fluorescent dye (final concentration 0.5 μM) and were equilibrated at 37°C for 15 min. Fluorescence was then monitored at

37°C under continuous stirring for 6 min. After registration for 3 min (resting conditions), gliosomes or synaptosomes were exposed to 0.5 μM ionomycin (stimulating conditions). Measurements were obtained within 2 h. Changes in fluorescence induced by ionomycin were calculated by subtracting the average of 60-s registration under resting conditions from the peak point of the curve obtained after exposure to the ionophore. Data were expressed as arbitrary fluorescence units.

Statistical analysis

The data reported were analysed using the two-tailed Student's *t*-test.

Results

Characteristics of the gliosome preparation

Gliosomes and synaptosomes were prepared from rat cerebral cortex tissue by homogenization and purification on a discontinuous Percoll® gradient. In order to verify the purity of our glial preparations, we performed laser confocal microscopy experiments. The images shown in Fig. 1 highlight the presence of GFAP and the Ca^{2+}-binding protein S-100 immunoreactivity in the particles present in purified gliosomes. Gliosomes were labelled with anti-GFAP or anti-S-100 and with anti-LDH antibodies. Because LDH is localized in the cytosol, it can be assumed that the LDH-positive particles represent re-sealed gliosomes or synaptosomes rather than membrane debris. The gliosome preparation efficiently stained for GFAP (Fig. 1a, green), S-100 (Fig. 1e, green) and LDH (Figs 1b and f, red) and merging the images revealed that the majority of the LDH-positive particles were also GFAP or S-100 positive (Figs 1c and g, white, respectively). Analysis of four different image couples indicated that nearly 90% of LDH co-localized with either GFAP (Fig. 1d) or S-100 (Fig. 1h). These figures also indicate that almost all GFAP- and S-100-positive particles

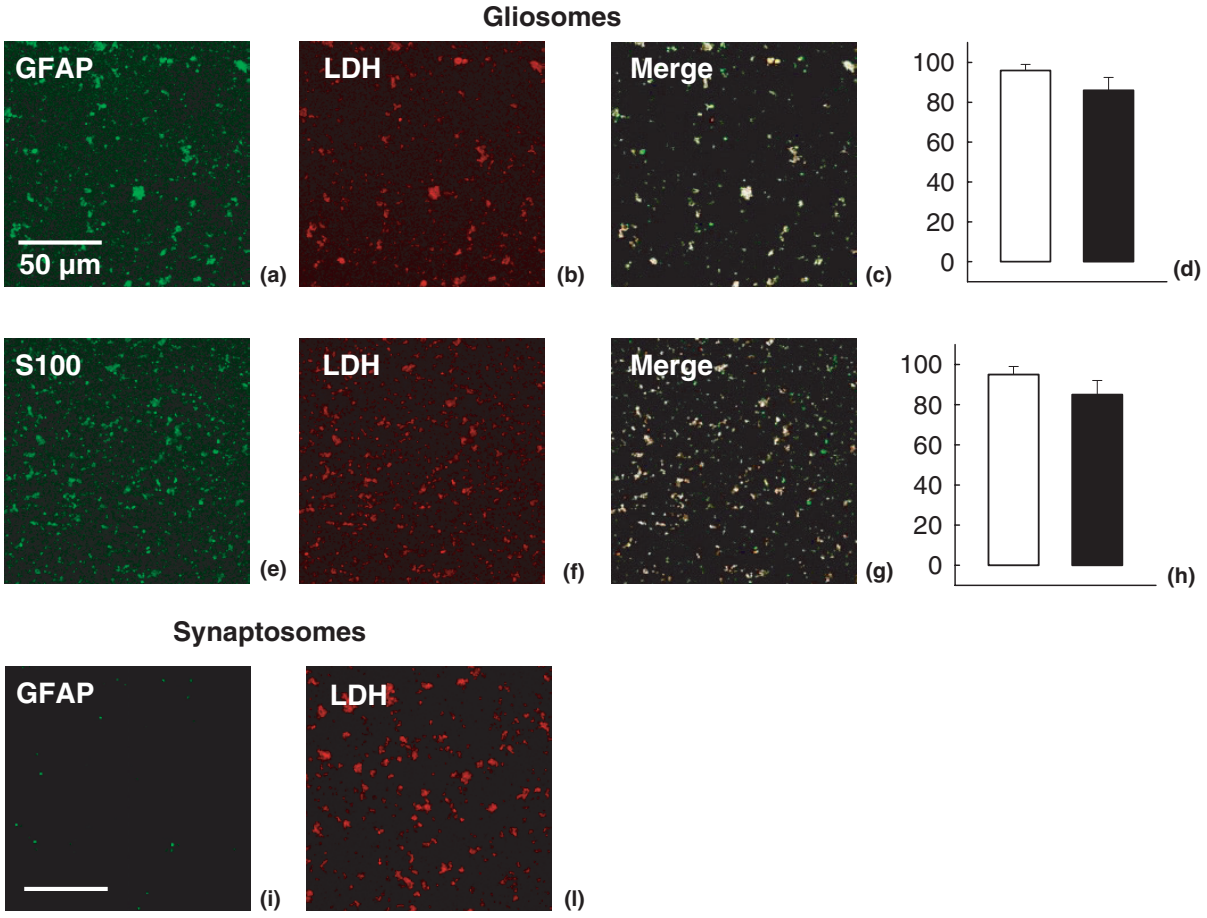


Fig. 1 Identification by immunocytochemistry of GFAP, LDH and the Ca^{2+}-binding protein S-100 in gliosomes and synaptosomes purified from rat cerebral cortex. Gliosomes and synaptosomes were glued on to coverslips, fixed with paraformaldehyde, permeabilized with Triton X-100, and incubated with the primary and secondary antibodies. Samples were analysed by laser confocal microscopy. Images show fluorescein-labelled anti-GFAP (a and i), Texas Red-tagged anti-LDH (b, f, and j) and fluorescein-tagged anti-S-100 (e). The white colour represents co-expression of GFAP and LDH (c) or of S-100 and LDH (g) in merged images. Bar plots show the co-localization of red in green (empty bars) and green in red (solid bars) of the couples GFAP/LDH (d) and S-100/LDH (h). Values are mean ± SEM of four different images.

were LDH positive. On the contrary, the synaptosomal preparation, which was extensively stained by LDH (Fig. 1j, red), did not show substantial GFAP labelling (Fig. 1i, green) indicating a low level of gliosome contamination.

In accordance with the data shown in Fig. 1, Fig. 2 shows that gliosomes, which were efficiently labelled by the GFAP antibody (Fig. 2a, d and g, red), showed only very modest if any, signal with antibodies raised against the neuronal markers PSD-95, MAP-2 or *β*-tubulin III (Fig. 2b, e and h, green), indicating that gliosomes represent a preparation with low synaptosomal contamination. Of note, antibodies against PSD-95, MAP-2 and *β*-tubulin III extensively marked synaptosomes under the same experimental conditions (Fig. 2c, f, and i, green).

The GFAP-expressing gliosomal preparation (Fig. 2j and l, red) did not exhibit labelling for either integrin αM or MBP, proteins selectively expressed in microglia and oligodendrocytes respectively (Fig. 2 k and m, green). In a control experiment, the antibodies used in confocal microscopy experiments revealed a single band of the appropriate molecular weight in western blot experiments, using proteins extracted from microglia primary cell cultures and from white matter of 19-day-old rats (L. Minghetti, personal communication).

Figure 2(h) shows a three-dimensional reconstruction at higher magnification of GFAP-positive gliosomal particles (green), which were also positive for LDH (not shown); these particles appeared to be spheroidal organelles of around 0.6 μm diameter with a propensity to cluster in physiological medium.

In order to gain more information on the characteristics of the gliosome preparation we performed electron microscopy experiments. Ultrastructural analysis indicated that the gliosome fraction exhibited morphological differences compared with synaptosomes (Fig. 3) First, the gliosome fraction contained fewer postsynaptic densities (compare Figs 3a and b). Analysis of different fields (total area 741 and 727 μm^2 for gliosomes and synaptosomes respectively) indicated that the number of postsynaptic densities amounted to $0.01/\mu m^2$ in the gliosome preparations and $0.06/\mu m^2$ in the synaptosome preparations. Second, several vesicles with a diameter of the membrane-bound area of approximately 30 nm and scattered within the cytoplasm were present in about 45% of the gliosomes. These vesicles (Fig. 3c) were either uncoated or clathrin coated, and did not show a clustered configuration, in contrast to findings in synaptosomes. Third, fewer mitochondria were associated with gliosomes than with synaptosomes: we counted 0.09 and 0.17 structures containing mitochondria per μm^2 in the gliosomal and synaptosomal fractions respectively (total areas of analysis as indicated above).

Ionomycin-induced glutamate release in gliosomes

When gliosomes and synaptosomes were pre-labelled with the non-metabolizable glutamate analogue [^{3}H]D-ASP and exposed in superfusion to a 90-s pulse of 0.5 μM ionomycin, a Ca^{2+}-selective ionophore that mediates Ca^{2+} influx without voltage-sensitive Ca^{2+} channel activation and previously shown to induce transmitter exocytosis from nerve terminals (Sanchez Prieto *et al*. 1987; Verhage *et al*. 1991), a substantial stimulus-evoked release of [^{3}H]D-ASP was measured (Fig. 4a). Under the present experimental conditions, ionomycin appeared to release a larger amount of [^{3}H]D-ASP from gliosomes than from synaptosomes. Interestingly, when used at a concentration as low as 0.1 μM, ionomycin selectively caused amino acid release from gliosomes. The release induced by 0.5 μM ionomycin was entirely dependent on the presence of external Ca^{2+}.

In order to rule out the possibility that the release observed represents an artefact resulting from use of the non-metabolizable glutamate analogue [^{3}H]D-ASP, we monitored the release of endogenous glutamate. Under resting conditions, the basal efflux of the excitatory amino acid amounted to mean ± SEM 773 ± 173 and 360 ± 48 pmol/mg protein for gliosomes and synaptosomes respectively. As shown in Fig. 4(b), ionomycin (0.5 μM; 90-s pulse) evoked release of endogenous glutamate from both preparations. The effects of ionomycin paralleled those described in the experiments with [^{3}H]D-ASP; more glutamate was released from gliosomes than from synaptosomes by the Ca^{2+} ionophore and release was strictly dependent on the presence of Ca^{2+} in the extracellular milieu. The involvement of vesicular glutamate release from gliosomes was supported by experiments with the vesicle membrane V-type ATPase inhibitor bafilomycin A1 (Bowman *et al*. 1988; Floor *et al*. 1990), which is expected to prevent the accumulation of the amino acid into vesicles (Moriyama and Futai 1990; Roseth *et al*. 1995). Gliosomes and synaptosomes were first incubated with bafilomycin A1 (0.5 μM) and then exposed in superfusion to 0.5 μM ionomycin. The effect of ionomycin on the release of endogenous glutamate was significantly decreased by bafilomycin A1 (Fig. 4b).

Exposure of gliosomes and synaptosomes to increasing concentrations of ionomycin (0.1, 0.5, 1 or 5 μM) resulted in a concentration-dependent increase in glutamate release by both preparations (Fig. 4c). The ratio of gliosomal to synaptosomal glutamate release changed by increasing the concentration of the ionophore. Ionomycin released the amino acid only from gliosomes when used at 0.1 μM; on the contrary, ionomycin released more glutamate from synaptosomes than from gliosomes when added at 5 μM.

The Ca^{2+} dependence of the ionomycin-evoked release of [^{3}H]D-ASP and endogenous glutamate from gliosomes and its sensitivity to bafilomycin A1 suggest that the ionophore-induced release is exocytotic in nature. In line with this, experiments performed with the fluorescent dye acridine orange (Zoccarato *et al*. 1999) showed that gliosomes and synaptosomes were able to accumulate the dye into acidic cytoplasmic organelles and that the application of 0.5 μM

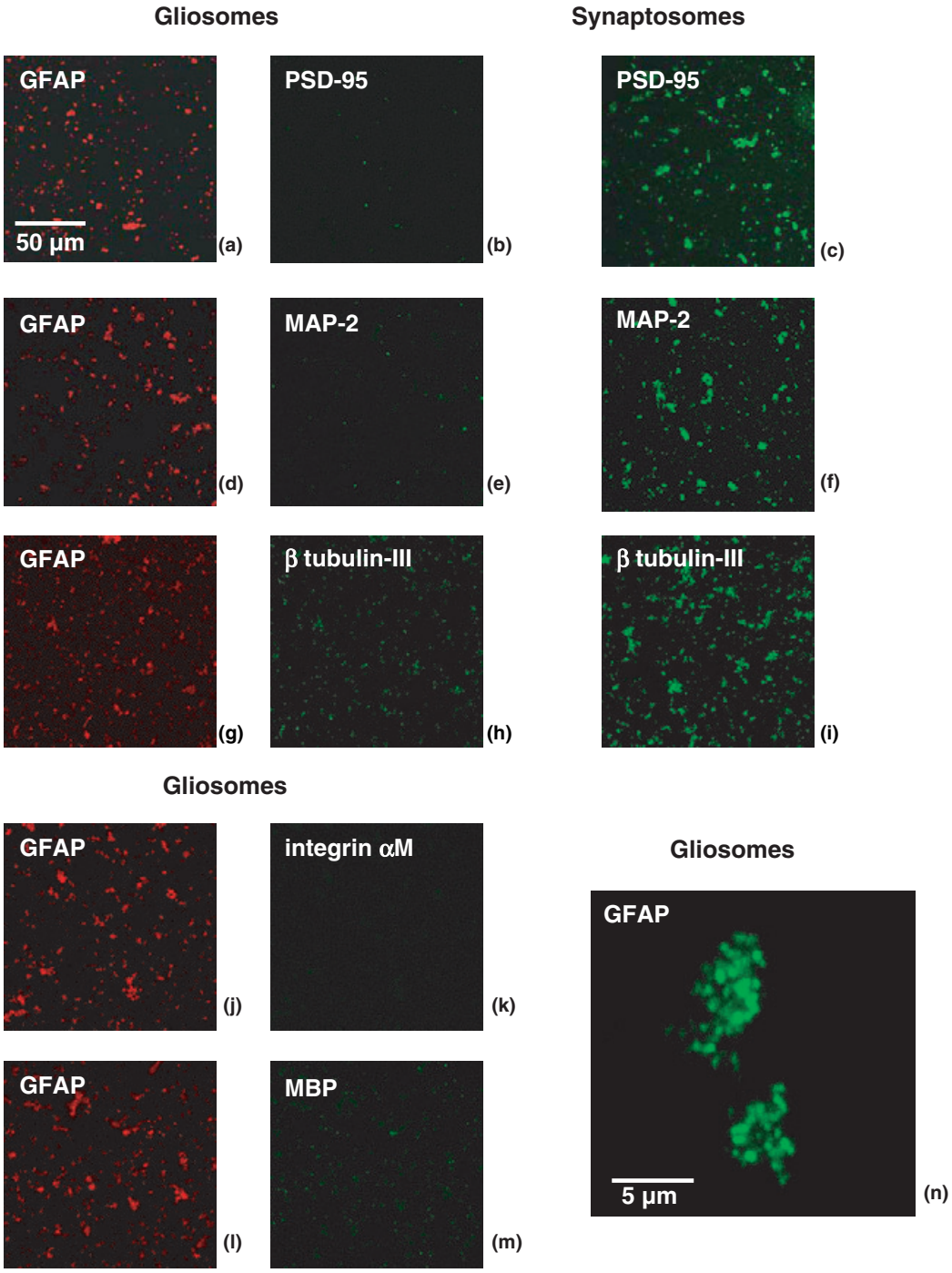


Fig. 2 Expression of glia- and neurone-specific proteins in gliosomes and synaptosomes. Gliosomes and synaptosomes were glued on to coverslips, fixed with paraformaldehyde, permeabilized with Triton X-100 and incubated with the primary and secondary antibodies. Samples were analysed by laser confocal microscopy. Images show Alexa Fluor 647-tagged GFAP (panels a, d, g, j and l). Alexa Fluor 488-tagged anti-PSD-95 (panels b and c), Alexa Fluor 488-tagged anti-MAP2 (panels e and f), Alexa Fluor 488-tagged anti-β-tubulin III (panels h and i), Alexa Fluor 488-tagged anti-integrin αM (panel k) and Alexa Fluor 488-tagged anti-MBP (panel m) in gliosomes purified from rat cerebral cortex. Panel n: high magnification three-dimensional reconstruction of purified gliosomal particles prepared from rat cerebral cortex and identified by immunocytochemistry.

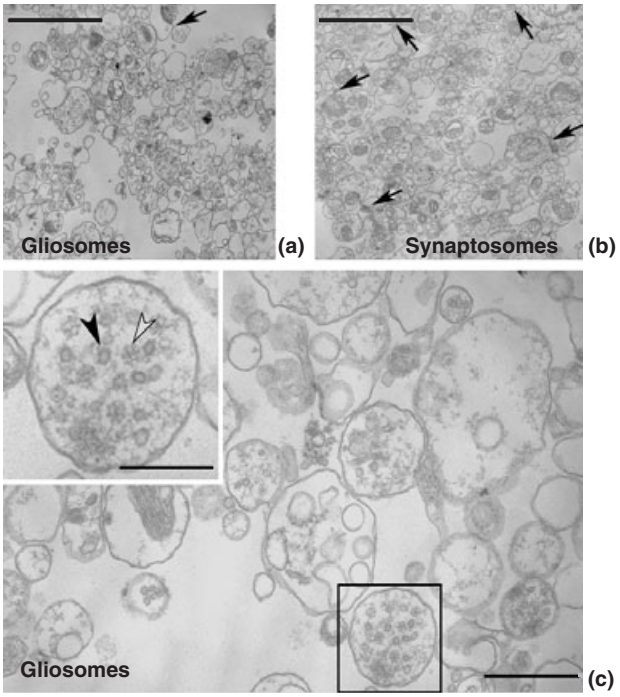


Fig. 3 Electron micrographs of gliosome (a and c) and synaptosome (b) fractions. Purified gliosomes and synaptosomes were fixed, dehydrated and embedded in LX112. Ultrathin sections were stained with uranyl acetate and lead citrate, and analysed with FEI CM10 or Tecnai 12 G2 electron microscopes. Low-magnification images highlight the lower number of synaptic densities (arrows) in gliosomes (a) than synaptosomes (b). Higher-magnification images (c) show a more detailed view of gliosomes. The black boxed area is reproduced at higher magnification in the insert and shows a gliosome containing approximately 30-nm smooth (black arrowhead) and clathrin-coated (white arrowhead) vesicles. Scale bar 2.25 μm (a); 2.25 μm (b), 0.54 μm (c), 0.28 μm (insert).

ionomycin induced Ca^{2+}-dependent fusion of these organelles with the plasma membrane. Figure 5 shows representative traces obtained with gliosomes (Fig. 5a) and synaptosomes (Fig. 5b). Analysis of four separate experiments highlighted changes in fluorescence build-up that were similar but not identical in the two preparations. In particular, the maximal effect in gliosomes was about 70% of that in synaptosomes (87 ± 6.8 and 122 ± 9.4 fluorescence units in gliosomes and synaptosomes respectively; $p < 0.05$) and the apparent kinetics of the phenomenon appeared to be appreciably slower in gliosomes than in synaptosomes (rise time 17.22 ± 3.13 and 2.11 ± 0.38 s in gliosomes and synaptosomes respectively; $p < 0.005$).

Traces in Fig. 5 show that the rise in fluorescence induced by 0.5 μM ionomycin was less pronounced in gliosomes than in synaptosomes. Conversely, Fig. 4 shows that the same concentration of ionomycin evoked a much greater glutamate release from gliosomes. This discrepancy can be explained easily by the fact that acridine orange signals reflect the exocytosis of many transmitters in synaptosomes whereas glutamate is likely to be the most abundant transmitter released by gliosomes.

ATP-induced glutamate release in gliosomes

To rule out the possibility that the exocytosis-like release of glutamate evoked by ionomycin might be linked to a unique characteristic of this agent, or result from unforeseen damage to gliosome integrity related to the use of the ionophore, we tested the effects of ATP, which has been reported to induce glutamate release from cultured astrocytes (Jeremic *et al*. 2001; Zhang *et al*. 2004b). Gliosomes were incubated in the presence of [^{3}H]D-ASP and superfused with increasing concentrations of ATP. As illustrated in Fig. 6, ATP (0.3, 1 or 3 mM) induced the release of [^{3}H]D-ASP in a concentration-dependent manner. ATP released a comparable amount of [^{3}H]D-ASP when applied to purified synaptosomes (data not shown). The effect of 3 mM ATP in gliosomes was significantly reduced by the selective P2 receptor antagonist PPADS at a concentration of 10 μM and abolished by 50 μM PPADS. The effect of ATP was only minimally affected in the absence of external Ca^{2+}, but was significantly diminished by pre-loading gliosomes with BAPTA.

Expression of proteins of the exocytotic machinery in gliosomes

Taken together, the release and fluorescence experiments are consistent with the idea that gliosomes can release glutamate by an exocytotic process. Accordingly, confocal microscopy experiments showed that purified gliosomes expressed the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which are known to form the core complex required to execute exocytotic neurotransmitter release in neurones (Südhof 1995). Gliosomes were labelled with anti-GFAP (Fig. 7a, e, i and m, red), anti-VAMP-2 (Fig. 7b, green), anti-syntaxin-1 (Fig 7f, green), anti-SNAP-23 (Fig. 7j, green) and anti-SNAP-25 (Fig. 7n, green). The preparation efficiently stained for all the proteins investigated. When images in the first and second panel of each row were merged, it appeared that a consistent portion of the GFAP-positive particles were also VAMP-2 positive .(Fig 7c), syntaxin-l positive (Fig. 7g), SNAP-23 positive (Fig. 7k) or SNAP-25 positive (Fig. 7o). The analysis of four to six different image couples indicated that about 55% of GFAP-expressing particles co-localized with VAMP-2 (Fig. 7d), about 70% of GFAP co-localized with both syntaxin-1 (Fig. 7h) and SNAP-23 (Fig. 7l) and about 40% of GFAP also co-localized with SNAP-25 (Fig. 7p). The percentage of the four SNARE proteins that co-localized with GFAP was between 84 and 92%. Similar experiments conducted by labelling gliosomes with anti-SNAP-23 and anti-SNAP-25 antibodies suggested that about 40% of SNAP-23 co-localized with SNAP-25, which in turn co-localized by about 70% with SNAP-23 (data not shown).

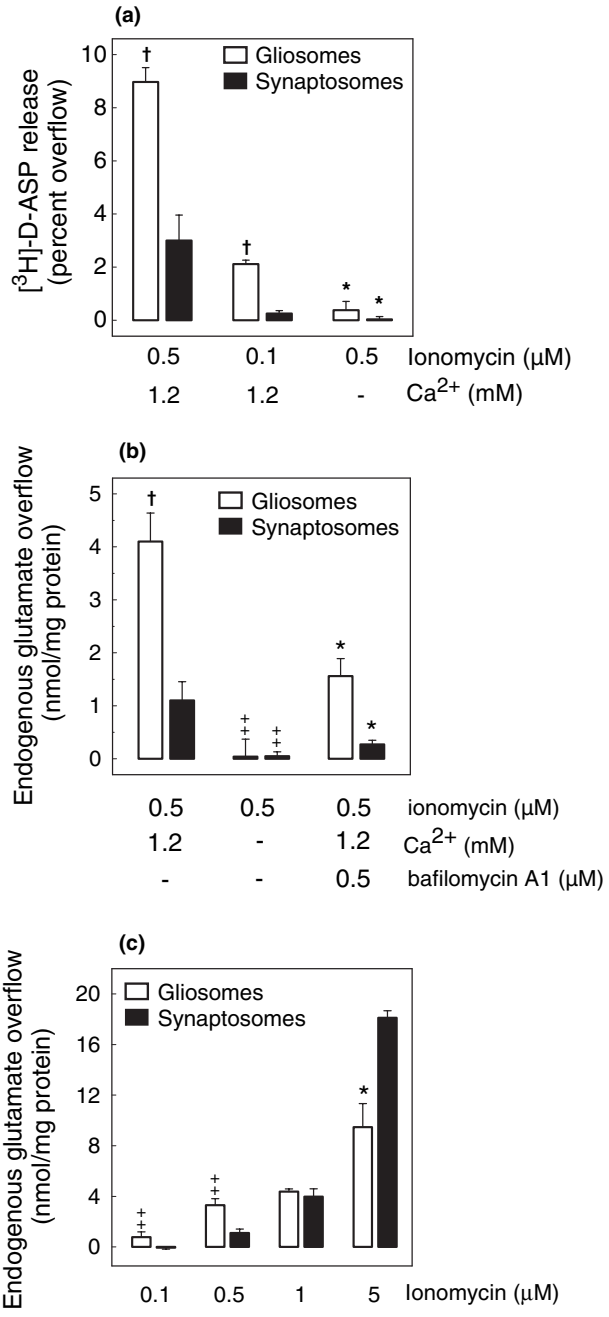


Fig. 4 Effect of ionomycin on the release of [^{3}H]D-ASP (a) or endogenous glutamate (b and c) from gliosomes and synaptosomes purified from rat cerebral cortex. Gliosomes and synaptosomes were incubated in the presence or absence of [^{3}H]D-ASP and exposed to a 90-s pulse of ionomycin at the concentrations indicated. When appropriate, the Ca^{2+}-free medium was introduced 19 min before ionomycin. Data are expressed as transmitter overflow. The stimulus-evoked transmitter overflow was calculated by subtracting the neurotransmitter content of basal release from the release evoked during and after exposure to ionomycin. (a) Tritium content in the fraction collected before depolarization amounted to 1.58 ± 0.15% of the total tritium tissue content in gliosomes and 0.82 ± 0.12% of that in synaptosomes ($n = 6$). Data represent the mean ± SEM of 5–6 separate experiments run in triplicate. The tritium overflow evoked by 0.5 μM ionomycin in gliosomes and synaptosomes and that evoked by 0.1 μM ionomycin in gliosomes was statistically different from the basal level ($p < 0.05$). *$p < 0.001$ versus respective overflow in medium containing 1.2 mM Ca^{2+}; †$p < 0.001$ versus respective overflow measured in synaptosomes (two-tailed Student's *t*-test). (b) In some experiments, gliosomes and synaptosomes were incubated for 30 min at 37°C in the presence or absence of 0.5 μM bafilomycin A1 before superfusion. Data represent the mean ± SEM of four separate experiments run in triplicate. All the overflows reported were statistically different from basal ($p < 0.05$), except for values in experiments using Ca^{2+}-free medium. †$p < 0.001$ versus respective glutamate overflow in synaptosomes; *$p < 0.01$, ‡$p < 0.001$ versus respective control glutamate overflow (two-tailed Student's *t*-test). (c) Data represent the mean ± SEM of four separate experiments run in triplicate. All the overflows reported were statistically different from basal ($p < 0.05$), except the values for 0.1 μM ionomycin in synaptosomes. *$p < 0.01$, ‡$p < 0.001$ versus respective glutamate overflow in synaptosomes (two-tailed Student's *t*-test).

Figure 7 also shows the data obtained with gliosomes labelled with GFAP, VAMP-2 and vGLUT-1 antibodies. The GFAP-expressing gliosomal preparation (Fig. 7q, red) also showed significant vGLUT-1 staining (Fig. 7r, green). The two proteins appeared to be partly co-localized (Fig. 7s, white). Analysis of four different image couples suggested that about 35% of GFAP co-localized with vGLUT-1 (Fig. 7t). VAMP-2 (Fig. 7u, red) and vGLUT-1 (Fig. 7v, green) were also co-expressed in gliosomes (Fig. 7w, white). About 65% of VAMP-2 co-localized with vGLUT-1 (Fig. 7x). Conversely, almost all of the vGLUT-1 was co-expressed with GFAP or VAMP-2.

Discussion

In the present paper we have shown that purified glial-derived organelles isolated from the adult rat brain, referred as to gliosomes, are able to take up glutamate and release it in an exocytotic mode.

Morphological evidence confirmed that the gliosomal fraction used here was minimally contaminated by synaptomes. Confocal microscopy experiments showed that the majority (about 90%) of LDH-labelled re-sealed particles in the gliosomal preparation were positive for GFAP or the Ca^{2+}-binding protein S-100, two proteins selectively expressed in astrocytes. These data suggest that a minority (about 10%) of particles in the purified gliosomal fraction are not of glial origin and possibly represent residual synaptosomes. The results shown in Fig. 7 support this conclusion: the percentage of SNARE proteins not co-expressed with GFAP, possibly synaptosomal SNARE proteins, ranged from 8 to 16%. Accordingly, three neuronal markers, namely PSD-95, MAP-2 and β-tubulin III, did not appreciably label the gliosomal fraction. Of note, gliosomes appeared not to be contaminated by other non-astrocytic glial elements because

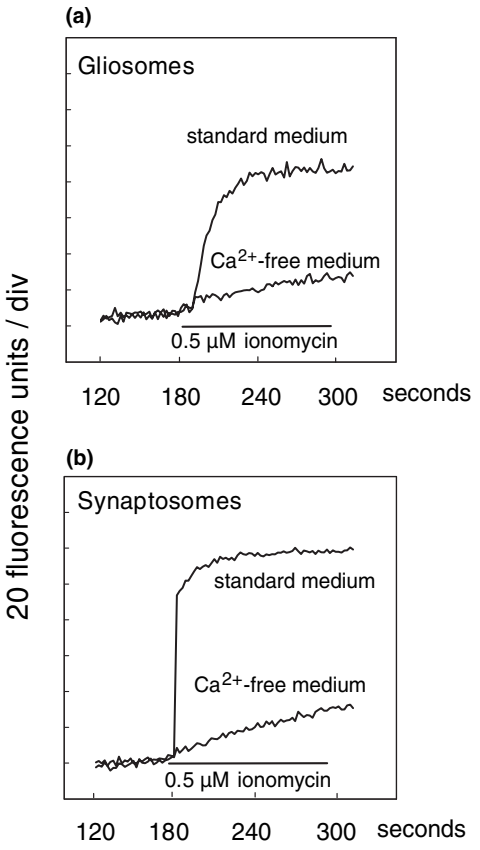


Fig. 5 Effect of ionomycin on vesicle membrane fusion in purified gliosomes (a) and synaptosomes (b) from rat cerebral cortex. Gliosomes and synaptosomes were resuspended in standard or Ca^{2+}-free HEPES-buffered physiological medium, as appropriate, and incubated at 37°C for 15 min in the presence of acridine orange before fluorometric determinations. Basal fluorescence levels were measured for 3 min, then 0.5 μM ionomycin was added and fluorescence recorded for a further 2 min. One-min basal fluorescence is reported in the figure. Fluorescence was recorded at 530 nm with excitation at 490 nm.

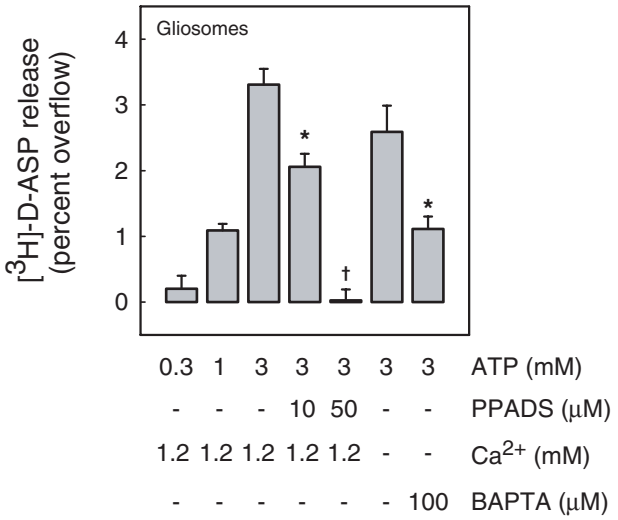


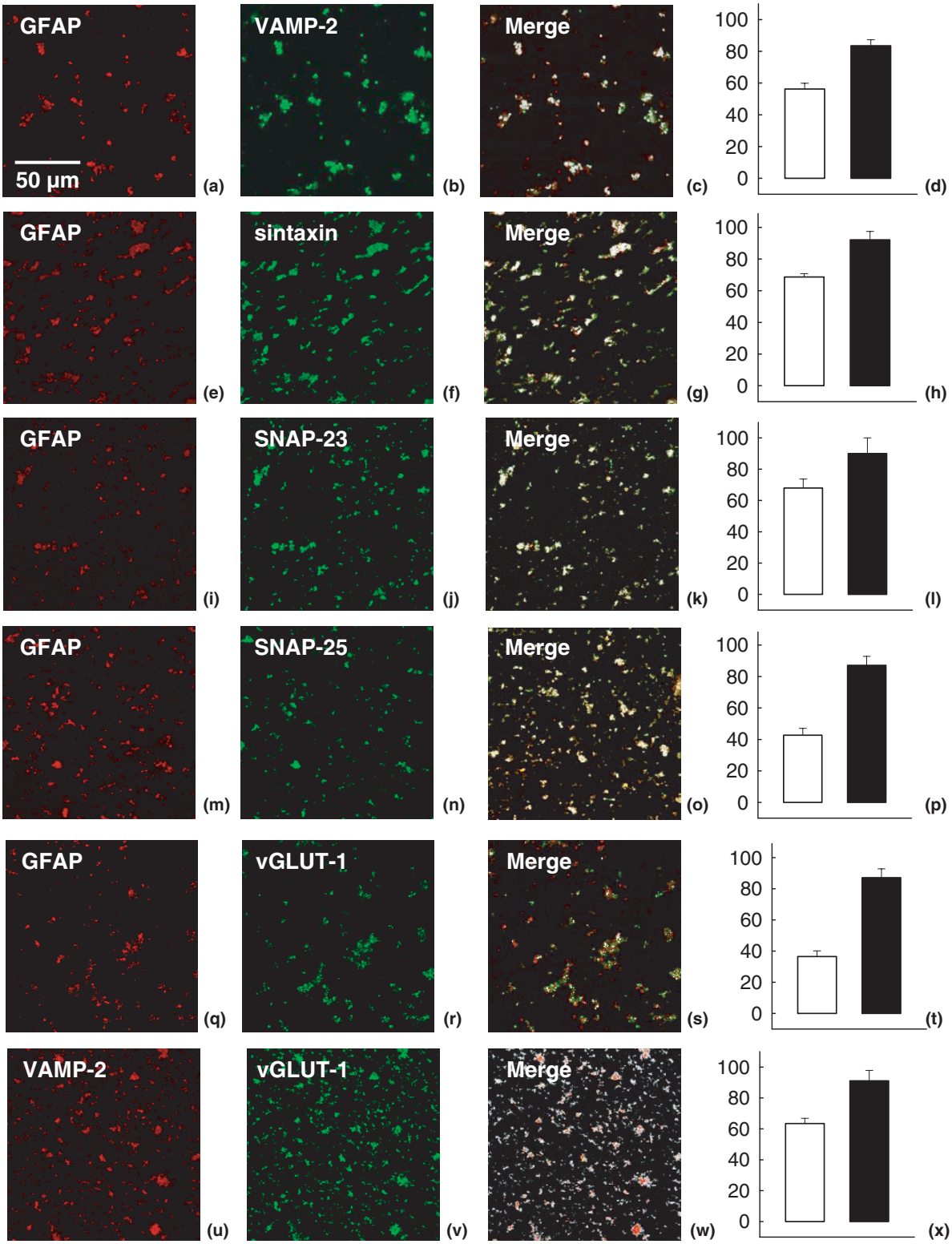
Fig. 6 Effect of ATP on the release of [^{3}H]D-ASP from gliosomes purified from rat cerebral cortex. Gliosomes were pre-labelled with [^{3}H]D-ASP, in the presence or absence of BAPTA-AM, and exposed to 0.3, 1 or 3 mM ATP at $t = 39$ min. ATP was present until the end of the experiments. PPADS (10 or 50 μM) was introduced 10 min before ATP. Ca^{2+} was omitted 19 min before addition of ATP. Data are expressed as transmitter overflow. The stimulus-evoked transmitter overflow was calculated by subtracting the neurotransmitter content of basal release from the release evoked during exposure to ATP. Data represent the mean ± SEM of 4–6 separate experiments run in triplicate. The tritium overflow evoked by 1 and 3 mM ATP was statistically different from basal ($p < 0.05$). *$p < 0.01$, †$p < 0.001$ versus respective ATP-induced overflow (two-tailed Student's *t*-test).

integrin αM and MBP, specific markers for oligodendrocytes and microglia respectively, were not present in the preparation. The release experiments testified to the high degree of purity of the gliosome and synaptosome fractions. In particular, low concentrations of ionomycin elicited glutamate release almost exclusively from gliosomes, whereas higher concentrations evoked release preferentially from synaptosomes. To conclude, gliosomes may represent a pure astrocyte-derived preparation, with limited interference by neuronal elements. In line with this view, ultrastructural analysis highlighted perceptible morphological differences between purified gliosomes and synaptosomes. In particular, gliosomes seemed not to bear postsynaptic densities and displayed non-clustered coated or smooth vesicles, whereas in synaptosomes the vesicles were often associated in grape-shaped clusters.

The experiments with ionomycin reported in Figs 4 and 5 suggest that gliosomes can release glutamate by a mechanism linked to an increase in cytosolic Ca^{2+} concentration and to

Fig. 7 Expression of proteins of the exocytotic machinery in the gliosomal fraction. Gliosomes were glued on to coverslips, fixed with paraformaldehyde, permeabilized -with Triton X-100 and incubated with the primary and secondary antibodies. Samples were analysed by laser confocal microscopy. Images show Alexa Fluor 647-tagged anti-GFAP (panels a, e, i, m and q). Alexa Fluor 488-tagged anti-VAMP-2 (panel b). Alexa Fluor 488-tagged anti-syntaxin-I (panel f). Alexa Fluor 488-tagged anti-SNAP-23 (panel j), Alexa Fluor 488-tagged anti-SNAP-25 (panel n), Alexa Fluor 488-tagged anti-vGLUTl (panels r and v) and Alexa Fluor 633-tagged anti-VAMP-2 -(panel, n). The white colour represents co-expression of GFAP and VAMP-2 (panel c), GFAP and syntaxin (panel g). GFAP and SNAP-23 (panel k) or GFAP and SNAP-25 (panel o), GFAP and vGLUT-I (panel s) and VAMP-2 and vGLUT-1 (panel w). Bar plots represent the expression of red in green (empty bars) and green in red (solid bars) of the couples GFAP/VAMP-2 (panel d), GFAP/syntaxin (panel h), GFAP/SNAP-23 (panel l), GFAP/SNAP-25 (panel p). GFAP/vGLUT-1 (panel t) and VAMP-2/vGLUT-I (panel x). Data represent the mean SEM of 4–6 different images.

exocytosis of glutamatergic vesicles. The release of the excitatory amino acid could also be induced by exposure of gliosomes to ATP. The glutamate overflow was linked to activation of specific P2 receptors and to an intraorganellar increase in Ca^{2+} concentration. The latter observation suggests, but does not actually prove, the exocytotic origin of the process. However, based on published findings that the release of glutamate induced by ATP in astrocyte cell



cultures is sensitive to bafilomycin A1 and abolished by preventing the formation of SNARE complexes (Jeremic *et al*. 2001; Zhang *et al*. 2004b), we can reasonably assume that the effects of ATP in gliosomes is linked to glutamate exocytosis induced by an increase in cytosolic Ca^{2+} concentration. Over the past 15 years several groups have reported that glial cells can exhibit a form a Ca^{2+} excitability, based on the occurrence of Ca^{2+} oscillations within astrocytes and their propagation to other contiguous glial cells (Enkvist *et al*. 1989; Parri *et al*. 2001; Peters *et al*. 2003; Cornell-Bell *et al*. 1990 Harris-White *et al*. 1998). There is now general agreement that glial cells, particularly astrocytes, respond to increases in intracellular Ca^{2+} by releasing agents that may mediate communication between glial cells and between glial and neuronal cells. One of these compounds is glutamate (Parpura *et al*. 1994; Araque *et al*. 1998), and recent reports suggest that the excitatory transmitter can be released from cultured astrocytes by an exocytotic process. The Ca^{2+} dependency of the process (Parpura and Haydon 2000), the ability of α-latrotoxin to induce glutamate release (Parpura *et al*. 1995b), and the sensitivity of the release to bafilomycin 1 and to clostridial toxins (Bezzi *et al*. 1998; Araque *et al*. 2000) support this conclusion. As to the mechanism of Ca^{2+}-dependent glutamate release, the most popular view is that glutamate release is driven by the interaction of various ligands with specific membrane receptors, whose activation leads to mobilization of Ca^{2+} from internal stores (Parpura and Haydon 2000; Bezzi *et al*. 2001, 2004; Pasti *et al*. 2001; Zhang *et al*. 2004b). Our results are compatible with glutamate being released from gliosomes by a Ca^{2+}-dependent exocytotic process, in line with published data obtained with fetal- or newborn-derived astrocytic cell cultures. Gliosome preparations may therefore be considered a useful model with which to study the functional properties of astrocytes *in situ*.

Several lines of evidence substantiate the ability of gliosomes to effect exocytosis of intraorganellar vesicles when subjected to stimuli that are able to augment the cytoplasmic Ca^{2+} concentration. The results of laser confocal experiments provided evidence for the presence of the SNARE proteins, known to be involved in vesicle fusion, in the purified gliosomal preparation, supporting the concept that gliosomes are capable of exocytotic release. Several studies have shown that cultured astrocytes express a number of proteins typically associated with synaptic vesicles or implicated in exocytosis, including those investigated in the present paper (Parpura *et al*. 1995a; Madison *et al*. 1996; Hepp *et al*. 1999; Maienschein *et al*. 1999; Volknandt 2002; Zhang *et al*. 2004a). It has been reported that the levels of some of these proteins may undergo drastic variation during *in vitro* cell maturation (Maienschein *et al*. 1999; Volknandt 2002). Very recently, SNAP-23 and VAMP-2 have been shown to be largely expressed in astrocytes *in situ*, with SNAP-25 being present only in a minority of cells (Wilhelm *et al*. 2004). The authors also demonstrated that the expression pattern of SNAP-25 increases when astrocytes were cultured *in vitro*. Accordingly, SNAP-23 and VAMP-2 were shown to be widely expressed in our glial preparation. At variance with the *in situ* data of Wilhelm *et al.* (2004), we found that appreciable amounts (about 40%) of gliosomes freshly purified from adult rat brain cortex express SNAP-25. It has been proposed that astrocytes possess specialized areas at the processes surrounding the synapses, by which they sense neuronal messengers and from which they in turn release gliotransmitters (Araque *et al*. 1999; Grosche *et al*. 1999; Carmignoto 2000). It might be speculated that the gliosomal preparation is enriched in these specific areas, where the release machinery of the glial cell, including SNAP-25, might be concentrated.

Expression of the vesicular integral protein VAMP-2 in a consistent portion of purified GFAP-positive astrocytic gliosomes suggests the presence of vesicles competent for fusion inside gliosomes. Of note, non-clustered vesicles of about 30 nm diameter were shown to be present in particles comprising the gliosomal fraction by ultrastructural analysis. This observation agrees with the recently reported presence of unpackaged vesicles within *in situ* astrocytes (Bezzi *et al*. 2004). In addition, the labelling of GFAP-positive gliosomes by a vGLUT-1 antibody suggests that these vesicles are glutamatergic in nature; however, the presence of vesicles storing other transmitters/modulators cannot be excluded if one considers the lower amount of vGLUT-1-positive gliosomes compared with VAMP-2-positive gliosomes (Fig. 7). It should be recalled, however, that the presence of type 2 and 3 vesicular glutamate transporters, which might be expressed by different sets of vesicles, was not tested for in the present study. Finally, the confocal microscopy experiments (Fig. 7a, panel iv) indicated that only about half of the GFAP-positive gliosomes were also positive for VAMP-2, suggesting that the remaining portion was not competent for exocytosis.

To conclude, gliosomes may represent a viable preparation that allows study of mechanisms of transmitter release and its regulation in adult astrocytes. Besides the obvious differences between gliosomes and intact cultured astrocytes, gliosomes may have a number of advantages: they can be prepared rapidly and, most importantly, originate directly from mature brain astrocytes. Gliosomes can be obtained from animals acutely or chronically treated with drugs, from knock-out or knock-down animals, from animals that represent models of brain diseases and from fresh human brain samples of surgical origin.

Acknowledgements

This work was supported by grants from Italian Ministry of University and Scientific Research (COFIN-MIUR 2002 and 2004 and FIRB 2003) to GB. We are grateful to Dr Luisa Minghetti

(Department of Cell Biology and Neurosciences, Istituto Superiore di Sanità, Rome, Italy), who performed control western blot analysis with the antibodies against integrin αM and MBP used in confocal microscopy experiments. We thank Ms. Maura Agate for her excellent secretarial assistance.

References

Angulo M. C., Kozlov A. S., Charpak S. and Audinat E. (2004) Glutamate released from glial cells synchronizes neuronal activity in the ippocampus. *J. Neurosci.* **24**, 6920–6927.

Araque A., Parpura V., Sanzgiri R. P. and Haydon P. G. (1998) Glutamate-dependent astrocyte modulation of synaptic transmission between cultured hippocampal neurons. *Eur. J. Neurosci.* **10**, 2129–2142.

Araque A., Parpura V., Sanzgiri R. P. and Haydon P. G. (1999) Tripartite synapses: glia, the unacknowledged partner. *Trends Neurosci.* **22**, 208–215.

Araque A., Li N., Doyle R. T. and Haydon P. G. (2000) SNARE protein-dependent glutamate release from astrocytes. *J. Neurosci.* **20**, 666–673.

Bergles D. E. and Jahr C. E. (1997) Synaptic activation of glutamate transporters in hippocampal astrocytes. *Neuron* **19**, 1297–1308.

Bezzi P. and Volterra A. (2001) A neuron–glia signalling network in the active brain. *Curr. Opin. Neurobiol.* **11**, 387–394.

Bezzi P., Carmignoto G., Pasti L., Vesce S., Rossi D., Rizzini B. L., Pozzan T. and Volterra A. (1998) Prostaglandins stimulate calcium-dependent glutamate release in astrocytes. *Nature* **391**, 281–285.

Bezzi P., Domercq M., Brambilla L. *et al.* (2001) CXCR4-activated astrocyte glutamate release via TNFa: amplification by microglia triggers neurotoxicity. *Nat. Neurosci.* **4**, 702–710.

Bezzi P., Gundersen V., Galbete J. L., Seifert G., Steinhauser C., Pilati E. and Volterra A. (2004) Astrocytes contain a vesicular compartment that is competent for regulated exocytosis of glutamate. *Nat. Neurosci.* **7**, 613–620.

Bowman E. J., Siebers A. and Altendorf K. (1988) Bafilomycins: a class of inhibitors of membrane ATPase from microorganisms, animal cells and plant cells. *Biochemistry* **85**, 7972–7976.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Carmignoto G. (2000) Reciprocal communication systems between astrocytes and neurones. *Prog. Neurobiol.* **62**, 561–581.

Cornell-Bell A. H., Finkbeiner S. M., Cooper M. S. and Smith S. J. (1990) Glutamate induces calcium waves in cultured astrocytes: long range glial signalling. *Science* **247**, 470–473.

Dani J. W., Chernjavsky A. and Smith S. J. (1992) Neuronal activity triggers calcium waves in hippocampal astrocyte networks. *Neuron* **8**, 429–440.

Enkvist M. O., Holopainen L. and Åkerman K. E. (1989) Glutamate receptor-linked changes in cytosolic Ca^{2+} and membrane potential in primary rat astrocytes. *Brain Res.* **500**, 46–54.

Fellin T., Pasqual O., Gobbo S., Pozzan T., Haydon P. G. and Carmignoto G. (2005) Neuronal synchrony mediated by astrocytic glutamate through activation of extrasynaptic NMDA receptors. *Neuron* **43**, 729–743.

Floor E., Leventhal P. S. and Schaeffer S. F. (1990) Partial purification and characterization of the vacuolar H^+-ATPase of mammalian synaptic vesicles. *J. Neurochem.* **55**, 1663–1670.

Grosche J., Matyash V., Moller T., Verkhratsky A., Reichenbach A. and Kettenmann H. (1999) Microdomains for neuron–glia interaction: parallel fiber signaling to Bergmann glial cells. *Nature Neurosci.* **2**, 139–143.

Harris-White M. E., Zanotti S. A., Frautschy S. A. and Charles A. C. (1998) Spiral intercellular calcium waves in hippocampal slice cultures. *J. Neurophysiol.* **79**, 1045–1052.

Haydon P. G. (2001) Glia: Listening and talking to the synapse. *Nat. Rev. Neurosci.* **2**, 185–193.

Hepp R., Perraut M., Chasserot-Golaz S., Galli T., Aunis D., Langley K. and Grant N. J. (1999) Cultured glial cells express the SNAP-25 analogue SNAP-23. *Glia* **27**, 181–187.

Jeremic A., Jeftinija K., Stevsanovic J., Glavavaski A. and Jeftinija S. (2001) ATP stimulated calcium-dependent glutamate release from cultured astrocytes. *J. Neurochem.* **77**, 664–675.

Madison D. L., Krüger W. H., Kim T. and Pfeiffer S. E. (1996) Differential expression of rab3 isoforms in oligodendrocytes and astrocytes. *J. Neurosci. Res.* **45**, 258–268.

Maienschein V., Marxen M., Volknandt W. and Zimmermann H. (1999) A plethora of presynaptic proteins associated with ATP-storing organelles in cultured astrocytes. *Glia* **26**, 233–244.

Meller R., Harrison P. J. and Elliott J. M. (2002) *In vitro* evidence that 5-hydroxytryptamine increases efflux of glial glutamate via $5\text{-}HT_{2A}$ receptor activation. *J. Neurosci. Res.* **67**, 399–405.

Mennerick S. and Zorumski C. F. (1994) Glial contributions to excitatory neurotransmission in cultured hippocampal cells. *Nature* **368**, 59–62.

Moriyama Y. and Futai M. (1990) H^+-ATPase, a primary pump for accumulation of neurotransmitters, is a major constituent of brain synaptic vesicles. *Biochem. Biophys. Res. Commun.* **173**, 443–448.

Nakamura Y., Iga K., Shibata T., Shudo M. and Kataoka K. (1993) Glial plasmalemmal vesicles: a subcellular fraction from rat hippocampal homogenate distinct from synaptosomes. *Glia* **9**, 48–56.

Nakamura Y., Kubo H. and Kataoka K. (1994) Uptake of transmitter amino acids by glial plasmalemmal vesicles from different regions of rat central nervous system. *Neurochem. Res.* **19**, 1145–1150.

Parpura V. and Haydon P. G. (2000) Physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons. *Proc. Natl Acad. Sci. USA* **97**, 8629–8634.

Parpura V., Basarasky T. A., Liu F., Jeftinija K., Jeftinija S. and Haydon P. G. (1994) Glutamate-mediated astrocyte–neuron signalling. *Nature* **369**, 744–747.

Parpura V., Fang Y., Basarsky T., Jahn R. and Haydon P. G. (1995a) Expression of synaptobrevin II, cellubrevin and syntaxin but not SNAP-25 in cultured astrocytes. *FEBS Lett.* **377**, 489–492.

Parpura V., Liu F., Brethorst S., Jeftinija K. V., Jeftinija S. D. and Haydon P. G. (1995b) Alpha-latrotoxin stimulates glutamate release from cortical astrocytes in cell culture. *FEBS Lett.* **360**, 266–270.

Parri H. R., Gould T. M. and Crunelli V. (2001) Spontaneous astrocytic Ca^{2+} oscillations *in situ* drive MNDAR-mediated neuronal excitation. *Nat. Neurosci.* **4**, 803–812.

Pasti L., Zonta M., Pozzan T., Vicini S. and Carmignoto G. (2001) Cytosolic calcium oscillations in astrocytes may regulate exocytotic release of glutamate. *J. Neurosci.* **21**, 477–484.

Perea G. and Araque A. (2005) Properties of synaptically evoked astrocyte calcium signal reveal synaptic information processing by astrocytes. *J. Neurosci.* **25**, 2192–2203.

Peters O., Schipke C. G., Hashimoto Y. and Kettenmann H. (2003) Different mechanisms promote astrocyte Ca^{2+} waves and spreading depression in the mouse neocortex. *J. Neurosci.* **23**, 9888–9896.

Porter J. T. and McCarthy K. D. (1996) Hippocampal astrocytes in situ respond to glutamate released from synaptic terminals. *J. Neurosci.* **16**, 5073–5081.

Raiteri L. and Raiteri M. (2000) Synaptosomes still viable after 25 years of superfusion. *Neurochem. Res.* **25**, 1265–1274.

Raiteri M., Angelini F. and Levi G. (1974) A simple apparatus for studying the release of neurotransmitters from synaptosomes. *Eur. J. Pharmacol.* **25**, 411–414.

Raiteri M., Sala R., Fassio A., Rossetto O. and Bonanno G. (2000) Entrapping of impermeant probes of different size into nonpermeabilized synaptosomes as a method to study presynaptic mechanisms. *J. Neurochem.* **74**, 423–431.

Roseth S., Fykse E. M. and Fonnum F. (1995) Uptake of 1-Glutamate into rat brain synaptic vesicles: effect of inhibitors that bind specifically to the glutamate transporter. *J. Neurochem.* **65**, 96–103.

Rutledge E. M. and Kimelberg H. K. (1996) Release of [^{3}H]-D-aspartate from primary astrocyte cultures in response to raised external potassium. *J. Neurosci.* **16**, 7803–7811.

Sanchez Prieto J., Sihra T. S., Evans D., Ashton A., Dolly J. O. and Nicholls D. G. (1987) Botulinum toxin A blocks glutamate exocytosis from guinea-pig cerebral cortical synaptosomes. *Eur. J. Biochem.* **165**, 675–681.

Südhof T. C. (1995) The synaptic vesicle cycle: a cascade of protein–protein interactions. *Nature* **375**, 645–653.

Verhage M., McMahon H. T., Ghijsen W. E. J. M., Boomsma F., Scholten G., Wiegant V. M. and Nicholls D. G. (1991) Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals. *Neuron* **6**, 517–524.

Volknandt W. (2002) Vesicular release mechanisms in astrocytic signalling. *Neurochem. Int.* **41**, 301–306.

Wilhelm A., Volknandt W., Langer D., Nolte C., Kettenmann H. and Zimmermann H. (2004) Localization of SNARE proteins and secretory organelle proteins in astrocytes *in vitro* and *in situ*. *Neurosci. Res.* **48**, 249–257.

Zhang Q., Fukuda M., Van Bockstaele E., Pascual O. and Haydon P. G. (2004a) Synaptotagmin IV regulates glial glutamate release. *Proc. Natl. Acad. Sci. USA* **101**, 9441–9446.

Zhang Q., Pangrsic T., Kreft M., Kržan M., Li N., Sul J., Salassa M., Van Bockstaele E., Zorec R. and Haydon P. G. (2004b) Fusion-related release of glutamate from astrocytes. *J. Biol. Chem.* **279**, 12 724–12 733.

Zoccarato F., Cavallini L. and Alexandre A. (1999) The pH-sensitive dye acridine orange as a tool to monitor exocytosis/endocytosis in synaptosomes. *J. Neurochem.* **72**, 625–633.

