Storage and uptake of D-serine into astrocytic synaptic-like vesicles specify gliotransmission

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Abstract

Glial cells are increasingly recognized as active players that profoundly influence neuronal synaptic transmission by specialized signalling pathways. In particular, astrocytes have recently been shown to release small molecules such as the amino acids L-glutamate and D-serine as "gliotransmitters", which directly control the efficacy of adjacent synapses. However, it is still controversial whether gliotransmitters are released from a cytosolic pool or by Ca^{2+}-dependent exocytosis from secretory vesicles, i.e., by a mechanism similar to the release of synaptic vesicles in synapses. Here we report that rat cortical astrocytes contain storage vesicles that display morphological and biochemical features similar to neuronal synaptic vesicles. These vesicles share some, but not all, membrane proteins with synaptic vesicles including the SNARE synaptobrevin 2 and contain both L-glutamate and D-serine. Furthermore, they show uptake of L-glutamate and D-serine that is driven by a proton electrochemical gradient. D-Serine uptake is associated with vesicle acidification and is dependent on chloride. While L-serine is not transported, serine racemase, the synthesizing enzyme for D-serine, is anchored to the membrane of the vesicles allowing local generation of D-serine. Finally, we reveal a previously unexpected mutual vesicular synergy between D-serine and L-glutamate filling in glia vesicles. We conclude that astrocytes contain vesicles capable of storing and releasing D-serine, L-glutamate, and most likely other neuromodulators in an activity-dependent manner.

Introduction

Glial cells and particularly astrocytes control synaptic strength and hence participate in normal and pathological brain function (Halassa and Haydon, 2010) through the regulated release of neuromodulators called gliotransmitters (Volterra and Meldolesi, 2005). The mechanisms for gliotransmitter release remain ill-defined. Two main routes have been proposed: 1) non-exocytotic release from cytosolic pools by membrane proteins, 2) Ca^{2+}-regulated exocytosis which requires the storage of gliotransmitters in secretory organelles (Volterra and Meldolesi, 2005; Halassa and Haydon, 2010; Hamilton and Attwell, 2010).

Among gliotransmitters, D-serine is recognized as an important signalling molecule that gates the activity of the synaptic N-methyl D-aspartate (NMDA) receptors in many brain areas (Mothet et al., 2000; Panatier et al., 2006; Henneberger et al., 2010; Fossat et al., 2012). Besides its physiological functions, D-serine promotes excitotoxicity when released in excess (Martineau et al., 2006). Hence, deletion of the biosynthetic enzyme, serine racemase, confers neuronal protection against ischemic and related cerebral injuries (Inoue et al., 2008; Mustafa et al., 2010).

Several lines of physiological evidence have suggested that glia may exocytose D-serine. Indeed, release is triggered by calcium rises and is inhibited by both tetanus neurotoxin and by blockers of V-ATPase (Mothet et al., 2005; Martineau et al., 2008). Also, D-serine immunoreactivity colocalizes with markers of the regulated secretory pathway such as the SNARE (soluble Nethylmaleimide-sensitive factor attachment protein (SNAP) receptor) synaptobrevin 2 (Sb2) (Martineau et al., 2008). Furthermore, tetanus neurotoxin injection or cytosolic calcium clamping suppressed long-term potentiation in the Schaffer collateral - CA1 pyramidal cell synapses (Henneberger et al., 2010); these inhibitory effects were fully reversed by adding D-serine. Interestingly, L-glutamate, another important gliotransmitter, undergoes Ca^{2+}- and SNARE-dependent release to modulate synaptic functions (Araque et al., 2000; Bezzi et al., 2004; Montana et al., 2004; Jourdain et al., 2007).

Nevertheless, the evidence for exocytotic release of D-serine and L-glutamate is mostly indirect and the exocytotic release hypothesis is confounded by the observation that glial cells can also release these gliotransmitters through non-exocytotic mechanisms (for reviews see Volterra and Meldolesi, 2005; Hamilton and Attwell, 2010). Thus, it cannot be excluded that tetanus neurotoxin or calcium clamping inhibits the regulated exocytotic insertion of transporters or ion channels into the plasma membrane, which are needed for (stimulated) release of gliotransmitters.

Another issue with the general acceptance for Ca^{2+}-regulated exocytosis of gliotransmitters is that the nature of the putative storage organelles is unknown. Furthermore, scant information exists about the mechanisms by which gliotransmitters are sequestered and stored. Therefore, obtaining such evidence is of fundamental importance for proving the concept of D-serine and L-glutamate exocytosis. Here we have carried out a detailed analysis of the contents and uptake features of Sb2-bearing secretory organelles isolated from astrocytes using multiple experimental approaches. We show that Sb2-positive glial secretory vesicles have morphological and biochemical features similar to synaptic vesicles (SVs) and store both L-glutamate and D-serine. Uptake of these gliotransmitters is energized by a proton electrochemical gradient generated by a V-ATPase and critically depends on chloride ions. Finally, we show that synthesis and vesicular uptake of D-serine are coupled and that a mutual synergy exists between D-serine and L-glutamate uptakes in astroglial vesicles.

Materials and Methods

Antibodies

Monoclonal antibody clones 69.1 against rat synaptobrevin 2 (Sb2) was previously characterized (Edelmann et al., 1995). The antibodies against the following proteins were used: cathepsin D (Santa Cruz biotechnology), cellubrevin (Covalab), D-serine (GemacBio), EEA1 (clone 14) and GM130 (clone 35) (Transduction Laboratories), Na^+/K^+-ATPase and SV2 (Developmental Studies Hybridoma Bank), PDI (clone RL90, Abcam), PMP70 (clone 70-18, Sigma), serine racemase (Santa Cruz Biotechnology and clone 29, BD Biosciences), synaptophysin (clone 7.2, Synaptic System), synaptotagmin 1 (clone 604.1, Synaptic System), TfR (clone H68.4, Invitrogen), vGlut1 and vGlut2 (Millipore), glutamine synthase (clone SH-B1, Sigma), 3-tubulin (clone TU-20, Chemicon) and glial fibrillary acidic protein (GFAP, DAKO). Rabbit polyclonal antibodies against Serine Racemase, SNAP23 and V-ATPase were kind gifts of Herman Wolosker (Technion-Israel Institute of Technology, Haifa, Israel), Thierry Galli (INSERM, Paris, France) and Nicolas Morel (CNRS, Gifsur-Yvette, France), respectively. Mouse monoclonal IgG1 was from Serotec (France). Secondary antibodies conjugated to horseradish peroxidase and Alexa Fluor 488 or 546 were obtained from P.A.R.I.S. and Invitrogen, respectively. Biotinylated donkey anti-rabbit antibody was from Jackson Immunoresearch and the goat anti-rabbit IgG coupled to 1.4 nm gold particles was from Nanoprobes.

Cell cultures

Cultured cortical astroglia cells were prepared from 0- to 4-day-old postnatal Wistar rats of either sex as previously described (Martineau et al., 2008). Cells were plated onto 150 cm² flasks, kept at 37 °C in a 5% CO_2 incubator for 14–20 days. Astroglia culture purity (>95%) was assessed by immunostaining against the astrocytic marker GFAP.

Organelle immunoisolation

All steps were carried out at 4–6 °C for western blot and transport analysis or at 0 °C for amino acid content analysis. For the immunoisolation of GVs, cultured astroglial cells were washed, harvested by scraping and resuspended in homogenization buffer (4 mM HEPES-KOH pH 7.4, 100 mM K_2-tartrate, 2 mM $MgCl_2$, protease inhibitor cocktail (Complete EDTA-free, Roche)). Cells were homogenized using a cell cracker (clearance 10 μm, European Molecular Biology Laboratory, Heidelberg, Germany) and the lysate was centrifuged for 10 min at 400 g. Low speed supernatants (LSS) (containing 1 mg/ml protein) were incubated with monoclonal antibodies against Sb2 for 2 h under rotation and with magnetic beads coupled to protein G (Ademtech) for one additional hour. The beads were further washed 3 times with homogenization buffer. Unbound membranes in the supernatant and LSS were pelleted by centrifugation at 175,000 g for 2 h in an 80Ti rotor. All membrane or bead pellets were resuspended in SDS-PAGE sample buffer before being processed for gel electrophoresis and immunoblotting with standard methods, or in appropriate buffer for further analysis. Isolations with murine IgG or at 21 °C were used as a control.

Immunoisolation of synaptic vesicles was performed following the same protocol, with the following modifications. The rat cerebral cortex of a male Wistar rat (2 months) was homogenized with a glass/Teflon homogenizer (10 strokes at 2,500 rpm) in homogenization buffer immediately after decapitation. The homogenate was centrifuged for 25 min at 35.000 g. The resulting supernatant (containing 3 mg/ml protein) was incubated 1 h with anti-Sb2 and 1 h with magnetic beads.

Electron microscopy on isolated vesicles

Immunobeads with bound organelles were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.4) for 1 h at room temperature. After a rinse in 0.1 M sodium phosphate (pH 7.4), the beads were embedded in 1% agarose in the same buffer. Agarose blocks were then postfixed for 1 h with OsO_4 (1% in phosphate buffer), dehydrated and embedded in Epon. During dehydration, they were stained en bloc with uranyl acetate in 50% ethanol. Thin sections (60 nm) were counter-stained with lead citrate and examined in a Philips 301 electron microscope.

Electron microscopy on brain slices

One month old Sprague-Dawley male rats were anesthetized (pentobarbital; 6 mg/100 mg body weight) and perfused intracardially with a mix of glutaraldehyde (2.5% or 0.5%) and paraformaldehyde (2% or 4%) in 0.1 M phosphate buffer (PB). For pre-embedding immunoperoxidase analyses, coronal brain vibratome sections (60 m) were pre-treated with a cryoprotectant solution (DMSO 20%, 2% glycerin in PB) then freeze-thawed by freezing in isopentane and thawing in PB. Sections were pre-treated with 1% $NaBH_4$, pre-incubated in 15% donkey serum (DS) in PBS, incubated with anti-D-serine polyclonal antibody (1:2000), and finally incubated in biotinylated donkey anti-rabbit antibody (1:500) in PBS. The sections were treated with an avidin-biotinylated peroxidase mixture (ABC kit, Vector) and peroxidase enzyme activity was then revealed using a 0.05% 3,3'-diaminobenzidine tetrahydrochloride solution added with 0.01% H_2O_2. Sections for pre-embedding immunogold were incubated along with those used for immunoperoxidase up to the second antibody stage, then incubated in goat anti-rabbit IgG coupled to 1.4 nm gold particles (1:100) and postfixed in 1% glutaraldehyde for 10 minutes followed by silver enhancement of the gold particles with an HQ Silver kit (Nanoprobes). The specificity of the D-serine staining was assessed by incubating some sections with a "saturated" anti-D-serine polyclonal antibody (previously preabsorbed with 500 μM of liquid-phase D-serine glutaraldehyde conjugate) (Puyal et al., 2006) or without primary antibody. Under these conditions, no immunoreactivity was detected. Sections were then postfixed in 1% OsO_4, dehydrated in graded ethanol series and flat-embedded in epoxy resin (Durcupan ACM, Fluka, Sigma-Aldrich) on glass slides. Ultrathin sections were done (70–90 nm), mounted on formvar-coated single slot grids, and stained with uranyl acetate followed by alkaline lead citrate. Ultrastructural analyses were performed in a Philips CM100 transmission electron microscope.

Acidification assay

Immunoisolated vesicles were eluted in 100 mM citrate pH 2.5 for 45 min and resuspended in acidification assay buffer 2x to get a final buffer composition of 10 mM HEPES-KOH pH 7.4, 4 mM KCl, 4 mM $MgSO_4$, and 0.32 M sucrose. The acidification assay was performed by measuring the fluorescence quenching of acridine orange (AO, Molecular Probes) (Maycox et al., 1988). The reaction was started by adding 10 mM MgATP in a stirred microcuvette containing 50 μl of vesicles and 20 μM AO, equilibrated at 32 °C. The reaction was stopped after 15–20 min by adding 50 mM $(NH_4)_2SO_4$ to dissipate the proton gradient across the vesicular membrane and release acridine orange from the vesicular lumen. Changes of fluorescence were analyzed using a Photon Technology International spectrofluorimeter (excitation: 490 nm, emission: 530 nm). The acidification kinetics were analyzed using Felix32 software (Photon Technology International) for acquisition of AO fluorescence and Prism4 (GraphPad) for kinetics and statistical analysis. Traces were normalized to the fluorescence at t = 0 and corrected for baseline shifts induced by volume changes associated with the additions.

Uptake of radiolabelled amino acids

Amino acids uptake inside immunoisolated vesicles was analyzed by a filtration assay with radiolabelled amino acids as described previously (Burger et al., 1991). 100 μl aliquots of Sb2-immunoisolated beads resuspended in assay buffer (0.32 M sucrose, 4 mM KCl, 4 mM MgSO$_4$ and 10 mM HEPES-KOH, pH 7.4) were incubated with 400 μM D-serine or 40 μM L-glutamate containing 4 μCi D-[^{3}H]serine or 2 μCi L-[^{3}H]glutamate (PerkinElmer). Uptake activities were assayed by rapid filtration on glass fibre filters (GF/C) using a cell harvester system (Connectorate AG) and by scintillation counting.

Determination of amino acid vesicular content

Immunoisolation was performed at 0 °C to prevent vesicular content leakage (Burger et al., 1991). Starting material and immunoisolates were treated with 5% (w/v) trichloroacetic acid to extract free amino acids as described (Hashimoto et al., 1992). Amino acids were determined by a laboratory-assembled capillary electrophoresis with laser induced fluorescence (CE-LIF) system (Scanlan et al., 2011) using pre-column derivatization with 10 mM naphthalene-2,3-dicarboxaldehyde (NDA) and 20 mM KCN for 20 min in the dark. Chiral separation and quantitation were achieved by employing a separation buffer consisting of 10 mM -cyclodextrin, 30 mM sodium dodecyl sulfate (SDS) and 75 mM borate buffer at pH 10.5. In addition, both a CE-LIF separation with non-chiral separation conditions (50 mM borate buffer at pH 8.42) as well as capillary electrophoresis-mass spectrometry (CE-MS) were used to verify analyte identifications and quantitation. The laboratory-assembled CE-MS instrument and separation conditions were as described previously (Lapainis, 2009; Nemes et al., 2011). Amino acid contents were corrected for nonspecific adsorption on beads determined by isolating vesicles at 21 °C, and normalized to Sb2 quantified by immunoblotting.

Immunostainings

Astrocytes cultured on glass coverslips were immunostained with pairs of antibodies as described previously (Martineau et al., 2008). Immunostainings of cerebral cortex with rabbit polyclonal anti-D-serine antibody was performed as described (Puyal et al., 2006) on 30 μm coronal brain sections of 2 month-old male rats. Images were acquired using a confocal microscope (Leica TCS SP2) and colocalization was quantified using Image J software as described (Martineau et al., 2008).

Statistical analysis

Uptake and acidification kinetics were analyzed using Prism4 software (GraphPad). Results are reported as mean ± SEM from 3–6 independent experiments. Statistical significance was defined at $P<0.05$ using the Student's t-test.

Results

Glial Sb2-containing vesicles are similar to synaptic vesicles

Although D-serine was originally shown to be present in brain astrocytes (Schell et al., 1995; Panatier et al., 2006; Williams et al., 2006), recent investigations have revealed that both D-serine and its synthesizing enzyme, serine racemase, are also present in neurons (Kartvelishvily et al., 2006; Miya et al., 2008). Therefore, to clarify the respective contribution of astrocytes and neurons in D-serine disposition in the mature rat cerebral cortex, we performed immunostainings for D-serine. Double immunostainings for D-serine with the astroglial marker glutamine synthase *in situ* revealed that D-serine is mainly present in the soma and processes of astroglia (Fig. 1*A*). The amino acid is uniformly distributed among glial cells while double immunostainings with the pan-neuronal marker 3-tubulin

indicated a moderate and uneven presence of D-serine in neuron soma and dendrites (Fig. 1A). Pre-embedding immunoperoxidase electron microscopy shows that D-serine is enriched in glial processes apposed to excitatory synapses while absent from presynaptic terminals and postsynaptic dendritic spines (Fig. 1B). Negative controls using a pre-absorbed anti-D-serine polyclonal antibody did not show any peroxidase staining (Fig. 1C).

Ca^{2+}-dependent exocytosis of gliotransmitters such as D-serine and L-glutamate requires their compartmentalization into secretory organelles. As mentioned above, we have previously shown that D-serine colocalizes with Sb2 (Martineau et al., 2008), raising the possibility that D-serine is stored in such vesicles within astrocytes. To test whether such vesicles are indeed capable of storing and/or transporting gliotransmitters, we immunoisolated Sb2-containing organelles from a low speed supernatant (LSS) of cultured astrocytes using beads coated with a monoclonal antibody specific for the cytoplasmic part of the SNARE protein Sb2 (Crippa et al., 2006; Martineau et al., 2008). Electron microscopy analysis revealed that the immunoisolated organelles mainly consist of small, clear vesicles with a homogeneous size distribution (40 ± 16 nm) (Fig. 2A,B). Thus, these isolated glial vesicles (GVs) are similar in size and morphology to GVs and SVs observed *in situ* (Harris and Sultan, 1995; Bezzi et al., 2004; Bergersen et al., 2012). Control beads coupled to non-immune IgG were devoid of bound membranes, suggesting that nonspecific contamination by other membranes is negligible. We next resolved the protein composition of immunoisolated GVs by immunoblotting (Fig. 2C–E). No signal for Sb2 was observed in control bead fractions. The immunoisolated organelles were devoid of the SV markers synaptophysin and synaptotagmin 1, excluding any contamination by SVs (Fig. 2D). GVs contain vesicular membrane proteins such as the SNARE protein cellubrevin, the V-ATPase, SV2 and the vesicular glutamate transporter type 2 (vGlut2) (Fig. 2E). The immunoisolates were virtually free of contamination by plasma membranes and other endomembranes (Fig. 2E) as evident from the absence of signals for Na^+-K^+ATPase and SNAP23 (plasma membrane), protein disulfide isomerase (PDI, endoplasmic reticulum), golgi matrix protein of 130 kDa (GM130, Golgi apparatus), early endosome antigen 1 (EEA1) and transferrin receptor (TfR) (endosomes), cathepsin D (lysosomes), and the 70 kDa peroxisomal membrane protein (PMP70) (peroxisomes).

Purified GVs contain endogenous D-serine and L-glutamate

We next investigated whether purified GVs contain amino acids. Capillary electrophoresis coupled to fluorescence detection revealed that GVs immunoisolated at 0 °C contained D-serine and L-glutamate in amounts that were much higher than in vesicles immunoisolated at 21 °C or in control incubations (Fig. 3, corrected vesicular amount of D-serine: 0.052 ± 0.005 nmol/μg Sb2, of L-glutamate: 0.076 ± 0.039 nmol/μg Sb2). This agrees with previous reports showing that amino acid neurotransmitters are only preserved inside synaptic vesicles when isolated at 0 °C (Burger et al., 1991). Although -aminobutyric acid (GABA) has been reported to be released by astroglia (Angulo et al., 2008), we could not detect significant amount of GABA in GVs suggesting its release from a cytosolic pool. To further confirm the identity of D-serine, we used conversion of D-serine by *Rhodotorula gracilis* D-amino acid oxidase (*Rg*DAAO) (Mothet et al., 2005) (Fig. 3A). The quantifications of serine and glutamate were also confirmed using capillary electrophoresis separation under non-chiral conditions followed by both fluorescence and mass spectrometry detections (Fig. 4).

To evaluate the physiological relevance of these results obtained on purified GVs, we investigated the presence of both GVs and D-serine on pre-embedded cerebral cortex preparations. Electron microscopy revealed the existence of clear round shaped organelles (i.e., vesicle-like structures) in glia processes (Fig. 5A). Pre-embedding immunogold stainings revealed that D-serine was associated with these vesicle-like structures in astrocytic processes facing asymmetric synapses (Fig. 5A). No immunogold particles were observed in

neuronal boutons (Fig. 5*A*) and in control conditions when brain slices were incubated with a pre-absorbed anti-ᴅ-serine polyclonal antibody (Fig. 5*B*).

Cortical neurons also have been reported to release ᴅ-serine (Kartvelishvily et al., 2006; Rosenberg et al., 2010). Therefore, SVs from the whole rat cerebral cortex were isolated following the same procedure. As expected (Takamori et al., 2006), Sb2-containing SVs express major SV proteins like synaptophysin, V-ATPase, and synaptotagmin 1 (Fig. 6*A*). In agreement with previous work (Burger et al., 1991), SVs contain ʟ-glutamate, glycine and GABA (Fig. 3 and 6*B*). However, ᴅ-serine was not detected although it is present in the cortex homogenate (Fig. 3 and 6*B*), supporting the notion that neurons do not exocytose ᴅ-serine but release it from a cytosolic pool (Kartvelishvily et al., 2006; Rosenberg et al., 2010). These results agreed with our observations that neuronal ᴅ-serine immunostaining was confined to the soma of neurons but was excluded from their distal neurites or boutons (Fig. 1 and 5).

Vesicular ᴅ-serine uptake is dependent on chloride concentration and is specific to astroglia

The presence of ᴅ-serine in GVs suggests that GVs possess a specific transporter for this gliotransmitter. Properties of vesicular transporters have been mainly studied in purified SV fractions. All of them are driven by a proton electrochemical potential (μH^+) generated by a V-ATPase (Edwards, 2007). We therefore examined whether GVs are capable of transporting ᴅ-serine in the presence of a proton electrochemical gradient. As shown in Fig. 7*A*, this is indeed the case. Immunoisolated GVs showed ATP-dependent uptake of ᴅ-[^{3}H]serine that was blocked by the V-ATPase inhibitor bafilomycin and sensitive to the uncoupler FCCP. Next, we tested whether the vesicles also transport glutamate as suggested by the presence of vGlut2 (Fig. 2) (Bezzi et al., 2004; Crippa et al., 2006). Again, ATP-dependent transport of ʟ-[^{3}H]glutamate was observed (Fig. 7*A*).

The proton electrochemical potential μH^+ has two components, the membrane electric potential ( ) and the pH gradient (pH). In SVs, the relative contribution of the two components is determined by the presence of chloride which provides charge balance to H^+ (Edwards, 2007). To characterize the dependence of ᴅ-serine uptake by GVs on the components of μH^+, we monitored vesicular acidification using the pH-sensitive dye, acridine orange (AO). Surprisingly, no ATP-dependent acidification was observed when Cl^- was added up to a concentration of 100 mM (Fig. 7*B*), in contrast to SVs that show strong acidification in the presence of chloride (Hell et al., 1990; Schenck et al., 2009). Apparently, GVs are impermeant to Cl^-. In contrast, addition of ᴅ-serine induced a dose-dependent acidification of GVs with a K_M of 6.9 mM for the initial rate of acidification (Fig. 7*C,D*). ᴅ-serine-dependent acidification as well as ᴅ-[^{3}H]serine uptake were, however, completely dependent on the presence of chloride, with a maximum at 4 mM chloride that then declined slightly at higher chloride concentrations (Fig. 7*E,F*). These results indicate that, at least under these conditions, chloride provides charge balance for the protons during the uptake of ᴅ-serine (which contains no net charge at neutral pH), suggesting that ᴅ-serine uptake is coupled to chloride transport. Replacement of K^+ by Na^+ and of SO_4^{2-} by gluconate shows no influence of these ions on the pH induced by ᴅ-serine (data not shown). The effect of chloride on the vesicular ᴅ-serine transport prompted us to assess the relative contribution of both components of the proton electrochemical potential μH^+ to the vesicular uptake of ᴅ-serine. The K^+ ionophore, valinomycin, and the K^+/H^+ exchanger, nigericin, selectively abolish or pH of μH^+, respectively. FCCP-sensitive ᴅ-[^{3}H]serine transport in GVs was reduced evenly to 35% by valinomycin or nigericin, indicating that both the electrical ( ) and chemical (pH) components of μH^+ are required for full activity of the vesicular ᴅ-serine transporter (Fig. 7*G*). The combination of valinomycin and nigericin dissipated both and pH resulting in an almost complete inhibition of the ᴅ-[^{3}H]serine uptake (Fig. 7*G*).

These results together with the role of chloride on D-serine transport strengthen our model in which D-serine and chloride uptake are coupled in GVs and, additionally, suggest an exchange of D-serine/chloride for protons. Contrary to L-glutamate, neither acidification nor transport are observed when D-serine is added to SVs (Fig. 8*A,B*), which in combination with the absence of D-serine inside SVs (Fig. 3, 5 and 6) demonstrates that the vesicular transport for D-serine is specific to GVs.

L-glutamate uptake in GVs and SVs share the same properties

The surprising properties of GVs with respect to acidification prompted us to investigate the properties of glutamate-induced acidification. In SVs, glutamate transport has been shown to be associated with acidification (Fig. 8*A*), suggesting that glutamate serves as counterion for proton transport (Maycox et al., 1988; Schenck et al., 2009). However, glutamate uptake is also dependent on the presence of chloride. Since chloride on its own induces strong acidification of SVs, it has been difficult to resolve to which extent the effects of chloride are direct (e.g., as activator of vGlut activity) or indirect by shifting the balance between   and   pH, and the role of chloride in activation and the transport cycle of vGluts is controversial (Schenck et al., 2009; Juge et al., 2010). Similar to SVs (Fig. 8*A*), glutamate induced acidification of GVs (Fig. 7*D*). The apparent K_M was 2.2 mM, similar to that reported for SVs (Naito and Ueda, 1985; Maycox et al., 1988), with the maximal rate being comparable to that induced by D-serine (Fig. 7*D*). Similar to D-serine, chloride has a biphasic effect on L-glutamate transport, with activation at low concentrations (4 mM) and inhibition at high concentrations (>50 mM) (Fig. 7*E*), resembling previous reports from SVs (Hell et al., 1990; Schenck et al., 2009). In contrast to D-serine-induced acidification, significant uptake was already measured in the absence of chloride. However, the inhibition at high chloride concentrations is weaker in GVs than that reported for SVs (Hell et al., 1990; Schenck et al., 2009). This discrepancy may be due to the lack of chloride permeability of GVs that thus cannot dissipate   , the component of the electrochemical potential fuelling glutamate uptake (Hell et al., 1990; Schenck et al., 2009).

D-Serine and L-glutamate vesicular loading exert mutual stimulation

D-Serine and L-glutamate are both stored and transported inside GVs. vGluts have been shown to enhance vesicular loading of GABA and monoamines in nonglutamatergic neurons through a process called vesicular synergy (for review, see El Mestikawy et al., 2011). Such vesicular synergy has not yet been described for gliotransmitters. Therefore, we investigated whether both amino acids could be present in the same GVs and to what extent L-glutamate is able to modulate the accumulation of D-serine inside GVs, and vice versa. To this end, the acidification induced by one amino acid was measured in the presence or absence of the other. Under standard conditions, the D-serine-induced acidification was unaffected by the presence of L-glutamate. On the contrary, 5 mM D-serine markedly accelerates to   280% the initial rate of acidification induced by L-glutamate (Fig. 9*A,B*). To determine if the vesicular acidification rate reflects the amino acid accumulation, we measured the transport of L-[^{3}H]glutamate in GVs for 10 min in presence of 10 mM D-serine, and vice versa. Consistent with the acidification assay, FCCP-sensitive L-[^{3}H]glutamate uptake by GVs was reproducibly increased by   350% in the presence of D-serine (Fig. 9*B*). Conversely, L-glutamate had a slightly smaller stimulatory effect (225%) on D-[^{3}H]serine uptake, which was not revealed by the indirect measure of the initial rate of loading. The interplay between D-serine and L-glutamate uptakes may reflect their common dependency on chloride. However, the influence on the loading rate and amplitude exerted by one amino acid on the other is not absolutely symmetrical. Since L-glutamate uptake relies mostly on   while D-serine uptake depends more equally on both   and   pH, this discrepancy may be due to their bioenergetic properties.

SVs are devoid of any transport activity for ᴅ-serine (Fig. 8). Therefore, in order to finally address whether the stimulation of vesicular ʟ-glutamate transport by ᴅ-serine is due to the presence or to the transport of the amino acid, we analysed the effect of 10 mM ᴅ-serine on ʟ-glutamate loading inside SVs. In contrast to GVs, ᴅ-serine had no effect on ʟ-glutamate uptake in SVs, excluding a direct action of ᴅ-serine on vGlut but rather suggesting a synergic effect of ᴅ-serine uptake on vGlut activity (Fig. 9*B*).

Uptake of newly synthesized ᴅ-serine is coupled to the activity of GV-associated serine racemase

To characterize the substrate stereoselectivity of ᴅ-serine transport, we investigated the effect of ʟ-serine on GV acidification. Addition of ʟ-serine induced acidification of GVs with an initial rate of acidification comparable to that of ᴅ-serine (Fig. 10*A,C*), suggesting that the vesicular transporter does not discriminate between the enantiomers. However, ʟ-serine is not retrieved in isolated GVs (Fig. 3). In addition, ʟ-serine did not further induce acidification when GVs were preloaded with ᴅ-serine, and vice versa (data not shown). We therefore tested whether ʟ-serine is first converted to ᴅ-serine by serine racemase (SR). While SR is reported to be principally cytosolic (Balan et al., 2009), confocal microscopy analysis revealed a particulate staining pattern, displaying significant colocalisation between SR and Sb2 in cultured astrocytes from rat brain cortex (20.6 ± 8.9% Sb2 *versus* SR, 25.2 ± 11.5% SR *versus* Sb2) (Fig. 10*B*), similar to the colocalization between ᴅ-serine and Sb2 (Martineau et al., 2008). Immunoblotting then revealed that SR is present in purified GVs (Fig. 10*B*) but absent from SVs (Fig. 8*C*). Thus, SR appears to be anchored to Sb2-containing GVs, suggesting that ʟ-serine is converted to ᴅ-serine that is then transported into GVs. Such a vesicular localization may guarantee local high concentrations of the substrate, which may be advantageous for efficient transport of this gliotransmitter. Indeed, incubation of GVs with a potent competitive inhibitor of SR, ʟ-erythro-3-hydroxyaspartate (HOAsp) (Strisovsky et al., 2005) strongly reduced ʟ-serine-induced acidification without affecting the acidification induced by ᴅ-serine (Fig. 10*C,D*). Since the acidification kinetics measured for ʟ-serine and ᴅ-serine without HOAsp are identical, neither newly synthesized nor pre-existing ᴅ-serine is taken up preferentially. Finally, similar to vGluts (Naito and Ueda, 1985), the transporter for ᴅ-serine is stereoselective.

Discussion

In this study we have isolated and characterized a population of small, clear vesicles from astrocytes with both morphological and biochemical features reminiscent of neuronal SV. We show that these vesicles contain the endogenous gliotransmitters ᴅ-serine and ʟ-glutamate and possess specific transport activities for these transmitters. We further characterized the properties of ᴅ-serine uptake and found that this transport activity is specific for astroglia, associated with vesicle acidification, dependent on chloride and is stereoselective. While ᴅ-serine synthesizing enzyme SR is present at the membrane of GVs, we observed a functional coupling between SR activity and ᴅ-serine vesicular transport. Finally, we provided evidence for an unexpected vesicular synergy between ᴅ-serine and ʟ-glutamate uptake in astrocytes. Together, our findings lend strong support to the view that both ʟ-glutamate and ᴅ-serine are released from astroglia by exocytosis from storage organelles with properties reminiscent of neuronal SVs.

The small vesicles immunoisolated from rat cortical astrocytes in culture share morphological and molecular properties with vesicles previously characterized from astrocytes *in situ* using immunocytochemistry and immunoelectron microscopy, notably their homogenous size (40 nm in diameter), round shape, clear appearance and presence of vGlut and SNARE proteins (Bezzi et al., 2004; Bergersen et al., 2012). It suggests that the vesicles isolated and characterized by us do indeed correspond to the GVs described earlier.

The morphological properties of GVs are also similar to those of synaptic vesicles (Harris and Sultan, 1995; Bergersen et al., 2012). Apparently, the two organelles also share some of the major proteins including Sb2, the V-ATPase, vGlut2 and SV2. However, other major SV proteins (such as synaptophysin and synapsins) are known to be absent from glial cells, and further work, involving quantitative comparisons of major proteins, is required to assess similarities and differences in the protein composition. Most importantly, the subcellular distribution clearly distinguishes GVs from SVs. According to electron microscopy analyses, GVs are far less abundant in astroglial processes than SVs in synaptic boutons. The dominance of SVs in the neuropil may explain why the presence of vesicular proteins in astroglia has been difficult to demonstrate *in situ* (Cahoy et al., 2008). The lower abundance of these organelles also required a modification of the immunoisolation protocol for GVs in comparison to SVs, involving upscaling of starting material, higher concentration of beads and coupled antibodies, and longer incubation times (see Materials and Methods).

In the present study, we show conclusively that two gliotransmitters, namely L-glutamate and D-serine, are stored inside GVs. However, these amino acids are retained by GVs only when isolated under appropriate conditions. Leakage of L-glutamate and D-serine can be prevented or at least minimized when vesicle isolation is carried out at 0 °C, in agreement with earlier reports on SVs (Burger et al., 1991). We cannot exclude that even under these conditions some efflux of amino acids occurred. The actual vesicular content may thus be higher than that found in our experiments. Since we do not know the number of Sb2 copies per GV, it is not possible to provide an accurate estimate of the intravesicular concentration of these amino acids. Presently, we cannot exclude the presence of other small organic molecules in GVs as indicated by unidentified peaks in the electropherograms.

This is the first demonstration of a specific vesicular transporter for D-serine with features reminiscent of, but not identical to, the well-established vesicular transporters for excitatory and inhibitory amino acids. D-serine is transported into GVs with an affinity similar to the affinity of the vesicular inhibitory amino acid transporter (VIAAT) for GABA (Christensen et al., 1991). Our work has revealed key features of D-serine vesicular transport: it is specific for astroglia, dependent on both components of μH^+ produced by a V-ATPase, associated with proton transport and requires chloride for activation. Since D-serine has no net charge at neutral pH and chloride is required for vesicular acidification induced by D-serine, we propose that chloride provides charge balance while being co-transported with D-serine in a coupled or uncoupled manner. A coupled transmitter/chloride co-transport mechanism has already been proposed for VIAAT (Juge et al., 2009). Alternatively, the vesicular D-serine transporter (VSERT) may harbour an uncoupled anion conductance activated by substrates, as reported for plasma membrane glutamate transporters (Fairman et al., 1995; Ryan and Mindell, 2007). Together, these data are compatible with a model in which the putative D-serine transporter operates as a D-serine/H^+ antiporter (Fig. 11). In addition, our study reveals that a spatial association of SR activity and D-serine vesicular transport results in a functional coupling between synthesis and uptake which has previously been described in SVs for GABA and dopamine (Chen et al., 2003; Jin et al., 2003; Cartier et al., 2010). The presence of SR at the membrane of GVs allows a local synthesis of D-serine and thus favors the efficient transport of the gliotransmitter. However, in contrast to GABA and dopamine (Chen et al., 2003; Jin et al., 2003), pre-existing D-serine is taken up into GVs without any preference over a newly synthesized one. Therefore, it is unlikely that the activity of SR directly influence the transport activity for D-serine. Brain SR does not possess a transmembrane region (Balan et al., 2009). This is also the case for the tyrosine hydroxylase and glutamate decarboxylase, the synthesizing enzymes for GABA and dopamine. Intriguingly, these enzymes are associated with the respective storage vesicles, apparently by means of a specific interaction with the respective transporters (Jin et al., 2003; Cartier et al., 2010). It is conceivable that vesicle binding SR is also mediated by an interaction with

the vesicular D-serine transporter, but for this issue to be addressed the transporter needs to be identified. Previously, binding of SR to the plasma membrane of astrocytes and neurons has been described (Balan et al., 2009; Mustafa et al., 2009). This binding occurs through interaction with phosphatidylinositol (4,5)-biphosphate (PIP2) in astrocytes (Mustafa et al., 2009) or through posttranslational modifications in neurons (Balan et al., 2009), and leads to inactivation of the enzyme. In contrast, our data show that vesicle-bound SR is active, suggesting that the binding mechanism is different. Inactivation of SR at the plasma membrane may be a means to stop conversion of L-serine at the "wrong" place, i.e., after incorporation of the GV membrane into the plasma membrane following exocytosis.

The presence of vesicular glutamate transporters and vesicular transport activity for L-glutamate has already been observed in astrocytes (Bezzi et al, 2004; Crippa et al., 2006; Bergersen et al., 2012). However, L-glutamate uptake inside GVs has not been fully characterized. In our study, we confirmed these previous reports and further analysed the uptake properties of this amino acid inside GVs. We demonstrated that L-glutamate is transported by GVs with an apparent affinity and a biphasic chloride dependency similar to that reported for SVs (Naito and Ueda, 1985; Maycox et al., 1988). However, in contrast to SVs we did not observe chloride-dependent acidification, which is difficult to reconcile with vGlut2 functioning as a chloride channel as recently suggested for vGlut1 (Schenck et al., 2009). The lack of a transport-independent chloride conductance compensating for proton transport is a major distinguishing feature of GV *versus* SV transport which requires further investigation.

The storage and transport of both D-serine and L-glutamate in GVs raises the question of their possible co-storage and thus co-release. Our vesicular content analyses (Fig. 3,4) do not allow us to distinguish between one population containing both gliotransmitters or two distinct populations dedicated to one gliotransmitter. Recently, immunogold colabelling of D-serine and L-glutamate in the adult hippocampus revealed that both amino acids are presumably stored in distinct vesicular pools (Bergersen et al., 2012). However the limited sensitivity of the immunogold technique does not allow unequivocal conclusion. Here, we report that D-serine transport stimulates L-glutamate uptake in GVs, indicating a functional cross-talk between the two transporters that can only be explained by both transporters residing on the same vesicle. The mechanism underlying this vesicular synergy between both amino acid uptakes requires further investigation. Whatever the mechanism, glial cytoplasmic D-serine when transported in GVs would be able to amplify L-glutamate-mediated gliotransmission. Interestingly, co-release of both gliotransmitters from the same vesicle would be a perfect combination for activating NMDA receptors. To our best knowledge, we provide the first experimental evidence for a cotransmitter action on vesicular glutamate uptake and extend the notions of both co-release and vesicular synergy beyond neurons. However, the existence of a population of GVs storing and releasing both D-serine and glutamate does not exclude the possibility of GVs loaded with one but not the other gliotransmitter.

In conclusion, our work supports the concept that astrocytes possess specific vesicles capable of sequestering and storing D-serine and L-glutamate as gliotransmitters, which undergo calcium-dependent exocytosis and modulate synaptic transmission. Additionally, the vesicular synergy unravelled in glial cells would represent a safe-cost metabolic process by reducing the energy demand of regulated exocytosis.

Acknowledgments

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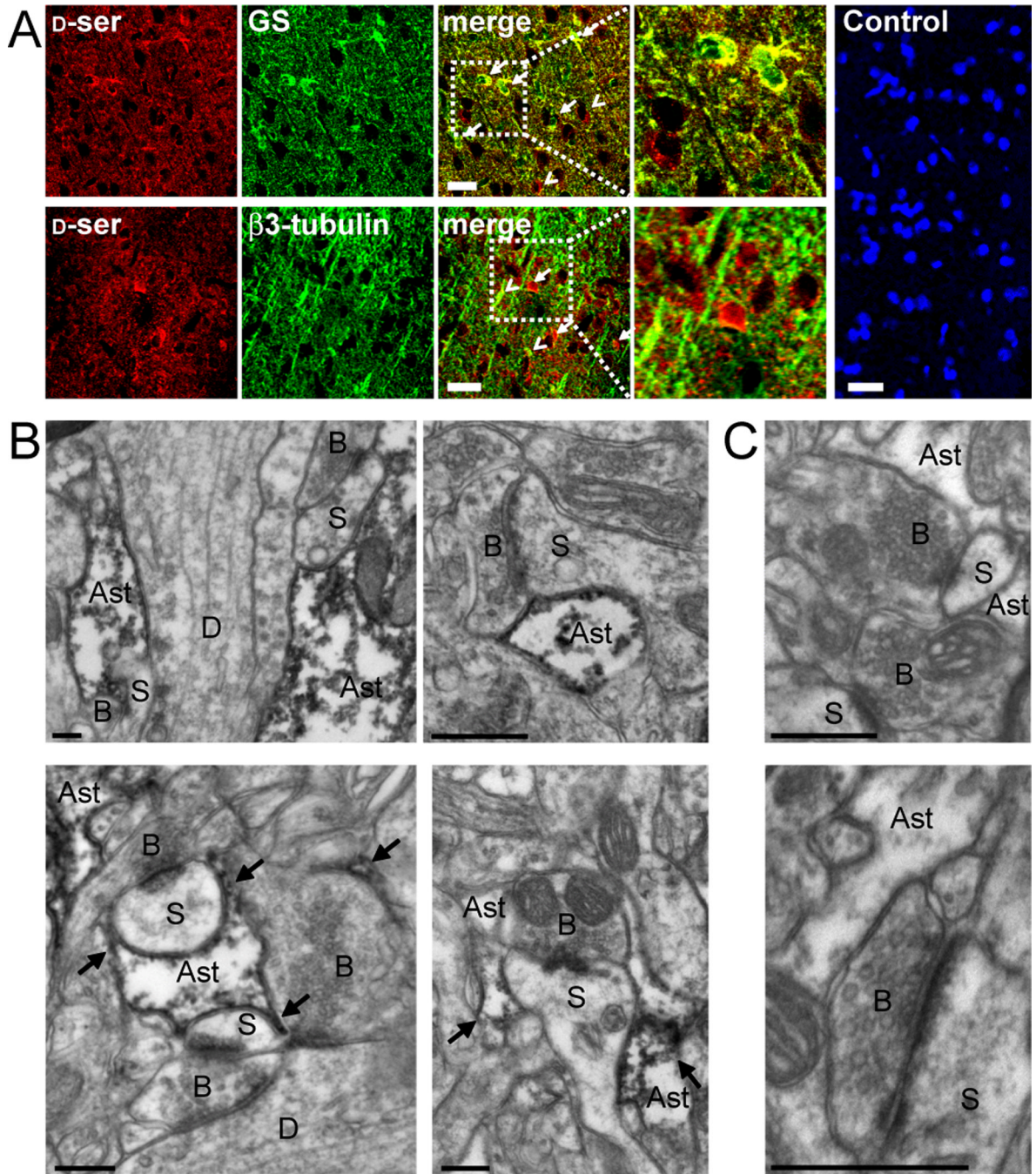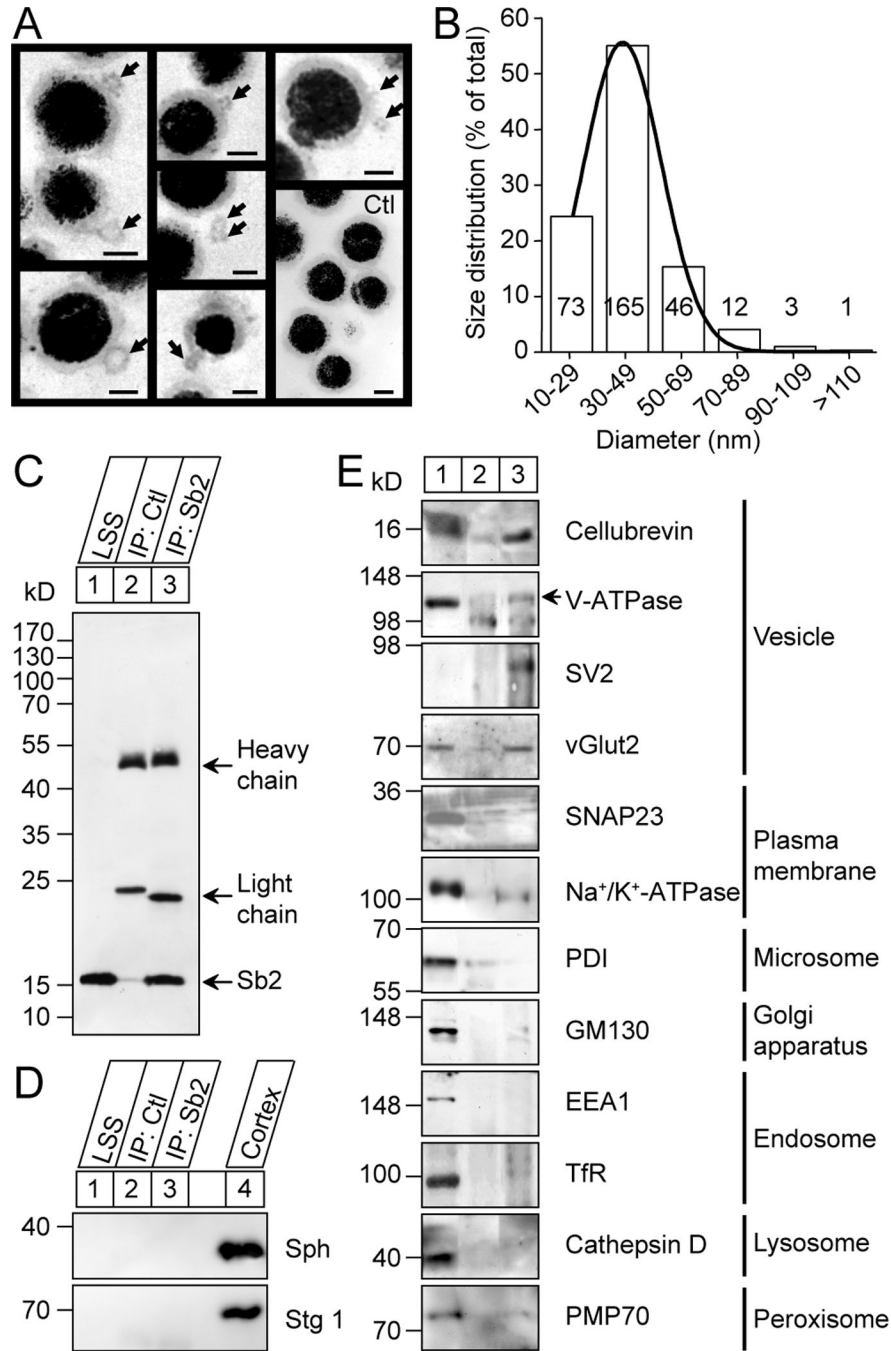
Figure 1.
ᴅ-serine distribution in the rat cerebral cortex. *A*, Confocal microscopy analysis of immunostainings of ᴅ-serine versus astroglial (glutamine synthase, GS) or neuronal (3-tubulin) markers in the rat cerebral cortex. Arrowheads: ᴅ-serine-containing neurons, arrows: ᴅ-serine positive astrocytes. Control: staining with a nuclear marker, DAPI (blue) and an anti-ᴅ-serine antibody previously pre-absorbed with 500 μM of liquid-phase ᴅ-serine glutaraldehyde conjugate. Scale bars: 30 μm. *B*, Representative electron micrographs of ᴅ-serine pre-embedding immunohistochemistry using the peroxidase method showing that ᴅ-serine immunoreactivity is mainly distributed in astrocytic processes (arrows) at the level of

asymmetric synapses in the rat cerebral cortex. *C*, Representative low magnification (upper panel) and high magnification (lower panel) electron micrographs showing that no peroxidase staining is detected with a pre-absorbed anti-ᴅ-serine antibody. Ast, astrocyte; D, dendrite; S, dendritic spine; B, axonal bouton. Scale bars: 500nm.

Figure 2.
Characterization of glial vesicles (GV), immunoisolated from cultured astrocytes using beads coated with anti-synaptobrevin 2 monoclonal antibodies. *A*, Electron micrographs of the bead fraction, showing vesicles bound to the surface of the beads. Ctl: Immunobeads coated with mouse IgG that were processed in parallel, no bound vesicles are detectable. Arrows: bead-bound vesicles. Scale bars: 100 nm. *B*, Size distribution of the immunoisolated vesicles. A total of 300 vesicles was measured, with the numbers falling into the respective categories indicated in the histogram. The black curve shows a Gaussian fit of the size distribution. *C*, Astroglial low-speed supernatant (LSS) and bead-bound

material (IP) were immunoblotted for Sb2. Equal proportion of samples was loaded showing that a major fraction of Sb2-positive organelles was recovered in the bead fraction. Light and heavy chains correspond to light and heavy chains of mouse control (lane 2) and monoclonal anti-Sb2 antibodies (lane 3). **D**, **E**, Immunoblots of the fractions shown in panel C for various marker proteins specific for SVs (**D**) and subcellular compartments (**E**). Sph: synaptophysin, Stg 1: synaptotagmin 1.
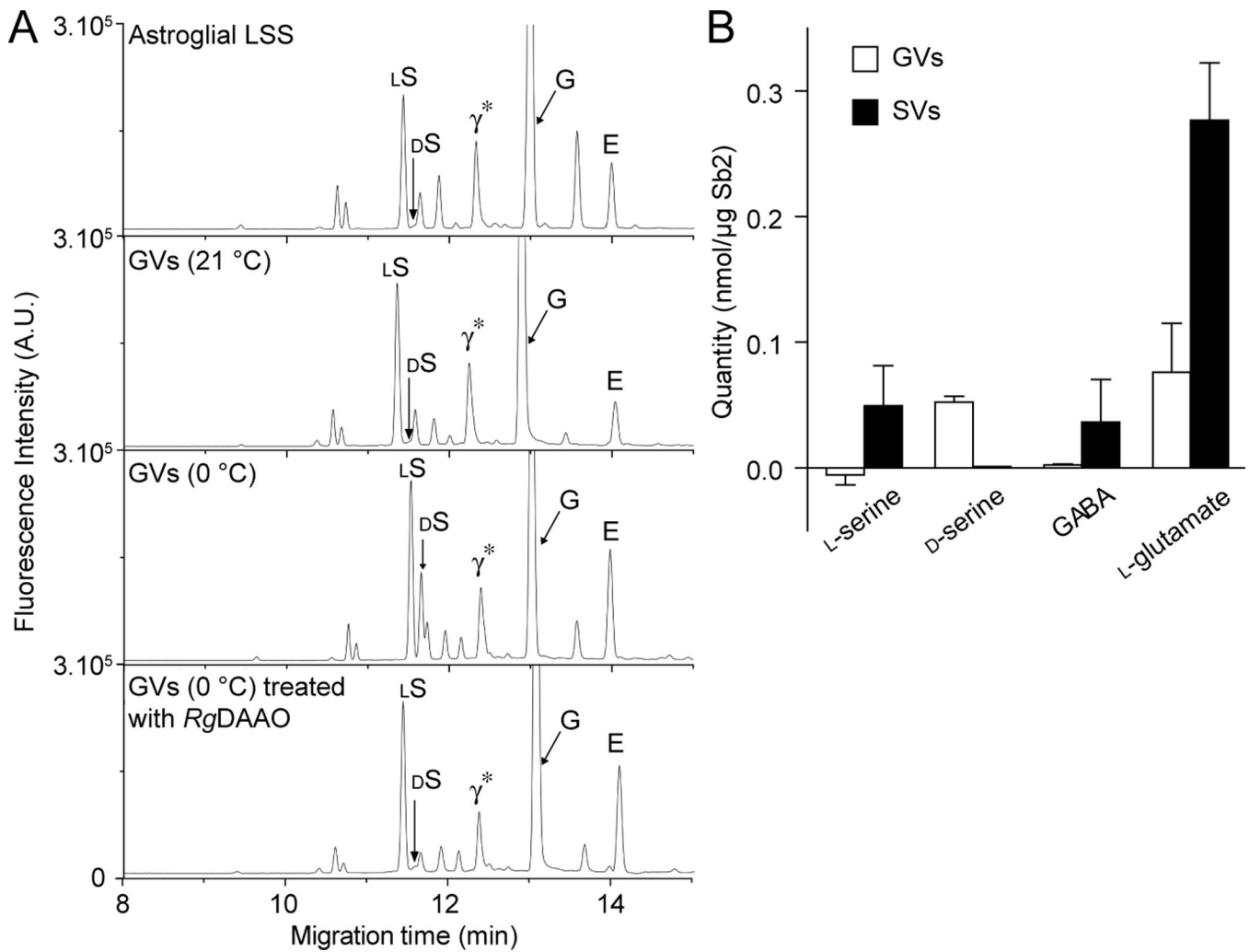
Figure 3.
Amino acid content of immunoisolated glial (GV) and synaptic (SV) vesicles. *A*, Astroglial low-speed supernatant (LSS), Sb2-containing vesicles immunoisolated at 21 °C and at 0 °C with or without *Rg*DAAO treatment were analyzed by capillary electrophoresis (CE). Electropherograms are adjusted to the same scale to facilitate the comparison between traces. Arrows point to the position of L-serine (LS), D-serine (DS), GABA + unknown substance (*), glycine (G) and L-glutamate (E). *B*, Quantification of amino acid content of GVs and SVs isolated at 0 °C. All values were corrected for nonspecific adsorption as determined by isolating vesicles at 21 °C. The figure shows mean values ± SEM of 4 independent experiments, normalized to synaptobrevin 2 as a vesicle marker.
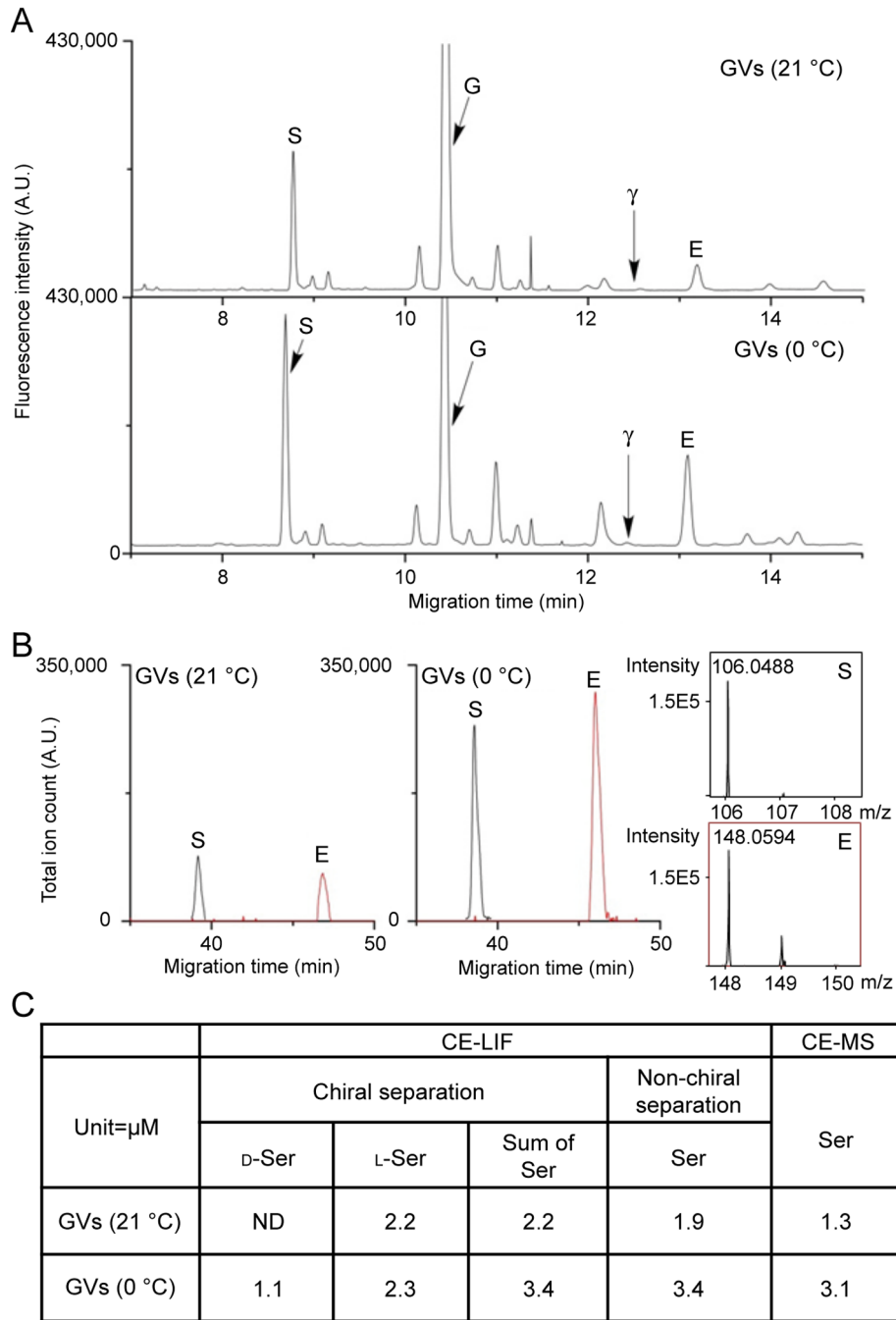
Figure 4.
Quantitation of vesicle amino acid content using CE-LIF and CE-MS. To validate
quantitation via CE-LIF chiral separations as shown in Fig. 3A, two additional
measurements using non-chiral separation conditions were performed using CE with laser
induced fluorescence (CE-LIF) detection and CE with mass spectrometric (CE-MS)
detection to validate the quantitative measures and peak assignments. *A*, Astroglial Sb2-
containing vesicles immunoisolated at 21 °C and at 0 °C were analyzed using a non-chiral
CE-LIF separation. Arrows point to the serine (S), GABA ( ), glycine (G) and glutamate (E)
peaks. *B*, The same samples were analyzed by CE-MS. Electropherograms were adjusted to

the same scale to facilitate comparison between approaches. *C*, A table shows representative quantification results of serine using these three different methods.
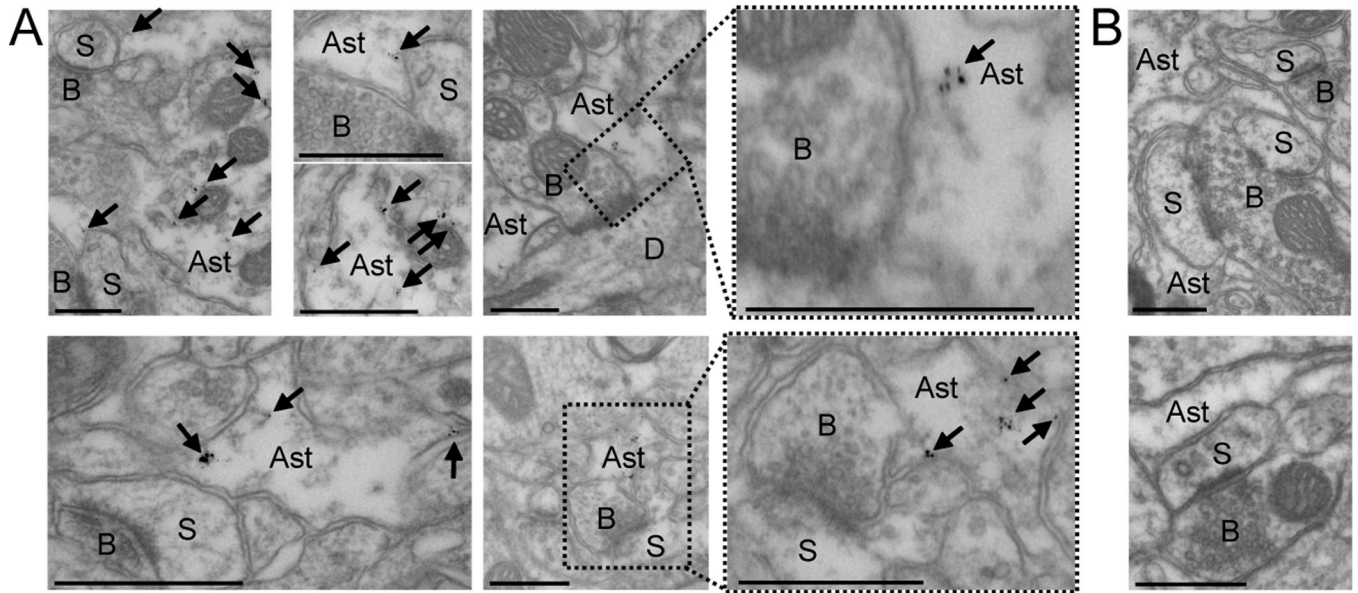
Figure 5.
Pre-embedding immunogold staining for D-serine at glia-neuron interfaces in rat cerebral cortex. *A*, Low magnification shows that immunogold-positive particles (arrows) are detected in astrocytic processes surrounding asymmetric synapses. High magnifications reveal that D-serine is associated with vesicle-like structures located in astrocytic processes facing the asymmetric synapses. Please note that the insets are rotated compared to the low magnification image. *B*, Representative low magnification (upper panel) and high magnification (lower panel) electron micrographs showing that no immunogold-positive particle is detected with a pre-absorbed anti-D-serine antibody. Ast, astrocyte; D, dendrite; S, dendritic spine; B, axonal bouton. Scale bars: 500 nm.
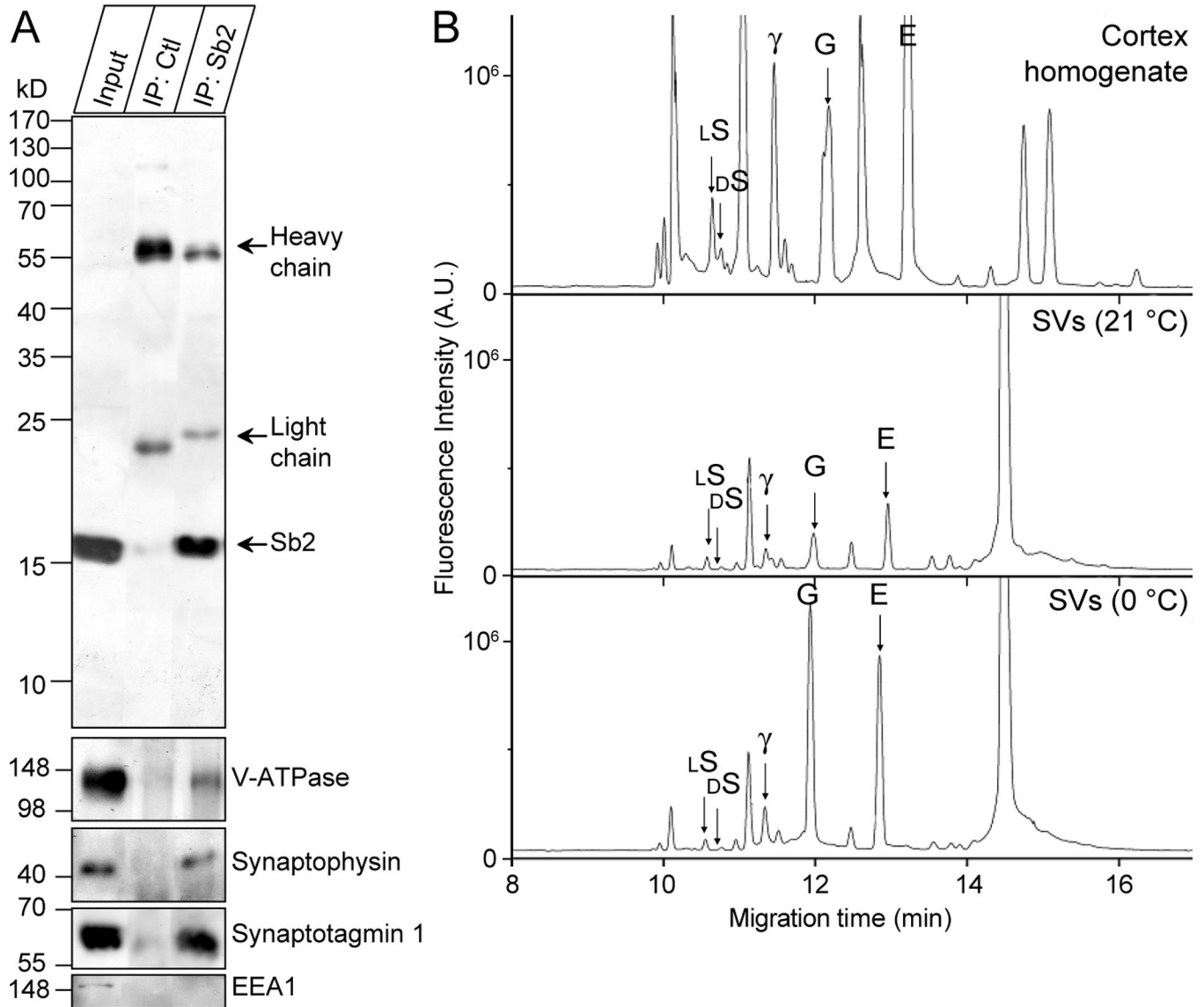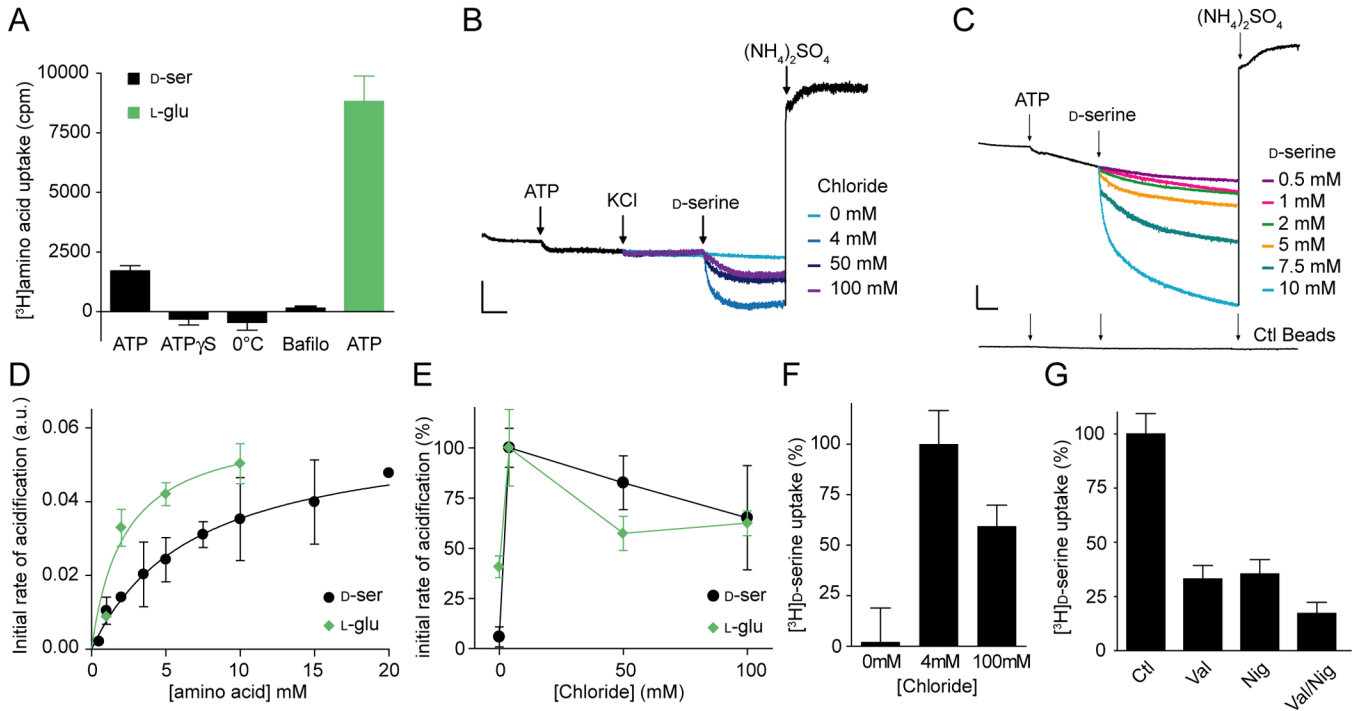
Figure 6.
Characterization of immunoisolated synaptic vesicles. *A*, Cortex homogenate (Input), and bead-bound material (IP) were immunoblotted for Sb2, V-ATPase (subunit a1), synaptophysin, synaptotagmin 1, and for the endosomal marker EEA1. Equal proportion of samples was loaded to allow direct comparison. Light and heavy chains correspond to light and heavy chains of antibodies. *B*, Cortex homogenate, Sb2-containing synaptic vesicles immunoisolated at 21 °C and at 0 °C were analyzed by CE-LIF. Electropherograms are adjusted to the same scale to facilitate the comparison between traces. Arrowheads point to the position of L-serine (LS), D-serine (DS), GABA ( ), glycine (G) and L-glutamate (E). Quantification of amino acid content in SVs is presented in Fig. 3*B* for comparison with GVs.

Figure 7.
Features of vesicular D-serine uptake into astrocytic immunopurified vesicles. **A**, ATP-dependent uptake of D-[^{3}H]serine and L-[^{3}H]glutamate. The values represent FCCP-sensitive uptake. FCCP (40 μM) was added together with MgATP (4 mM). Control experiments are performed by replacing MgATP by ATPγS (4 mM), by decreasing temperature to 0 °C, or by inhibiting V-ATPase with bafilomycin A1 (1 μM). **B** D-Serine (7.5 mM) mediated acidification of GVs is dependent on chloride (the figure shows representative traces from 3–6 independent measurements). To maintain ionic strength and osmolarity, chloride was exchanged for gluconate (potassium salts). Arrows correspond to addition of the tested compound (ATP 10 mM and (NH$_4$)$_2$SO$_4$ 50 mM, final concentrations in this and the following figures). At the end of the reaction, (NH$_4$)$_2$SO$_4$ was added to dissipate ΔpH. **C**, Overlay of photometric traces showing acidification of GVs induced by various D-serine concentrations in presence of 4 mM chloride (representative of 3–5 independent experiments). No change in fluorescence under any condition was observed when control beads (mouse IgG) were used. Scale bars represent 2 a.u. per 1 min. **D**, Dependence of the initial rate of acidification on D-serine and L-glutamate concentration. **E, F**, Initial rate of acidification induced by D-serine (7.5 mM) or L-glutamate (5 mM) and D-[^{3}H]serine uptake were measured in the presence of different chloride concentrations. **G**, Influence of both components of the proton electrochemical potential ΔμH$^+$ on FCCP-sensitive ATP-dependent D-[^{3}H]serine uptake. Additions: 5 μM nigericin, 20μM valinomycin. Error bars represent mean ± SEM; n = 3–6 independent experiments.
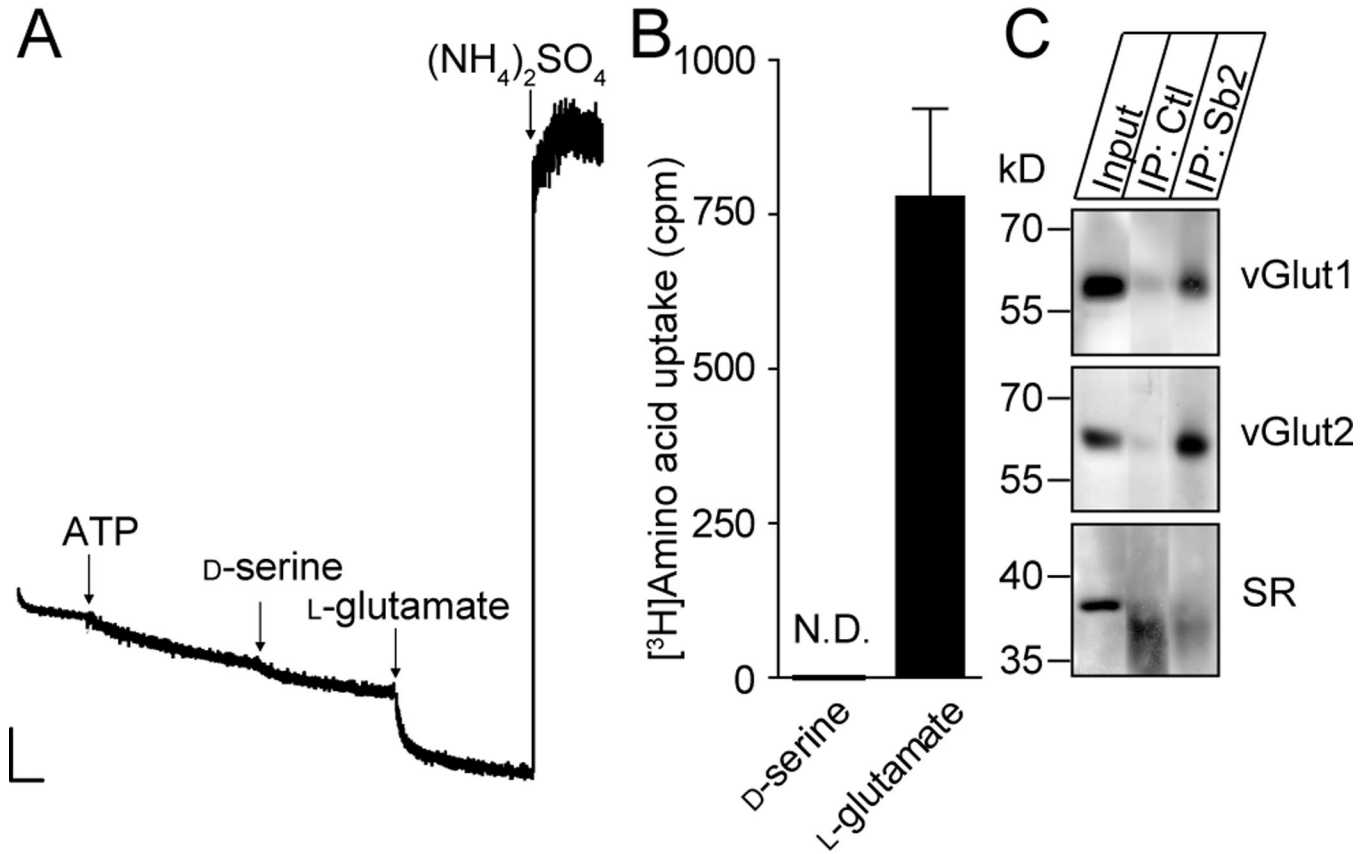
Figure 8.
Synaptic vesicles transport L-glutamate, but not D-serine. *A*, Photometric trace showing the absence of D-serine-induced acidification of synaptic vesicles whereas L-glutamate can acidify these organelles (10 mM each, n = 3). Scale bar: 2 a.u. per 1 min. *B*, ATP-dependent uptake of D-[³H]serine and L-[³H]glutamate by isolated SVs. The values represent FCCP-sensitive uptake. N.D. not detectable. Values represent mean ± SEM of 2 independent experiments performed in triplicate. *C*, Western blot analysis of cortex homogenate (Input) and control or Sb2 immunoisolates (IP) for vesicular glutamate transporters vGlut1 and vGlut2 and for serine racemase (SR).
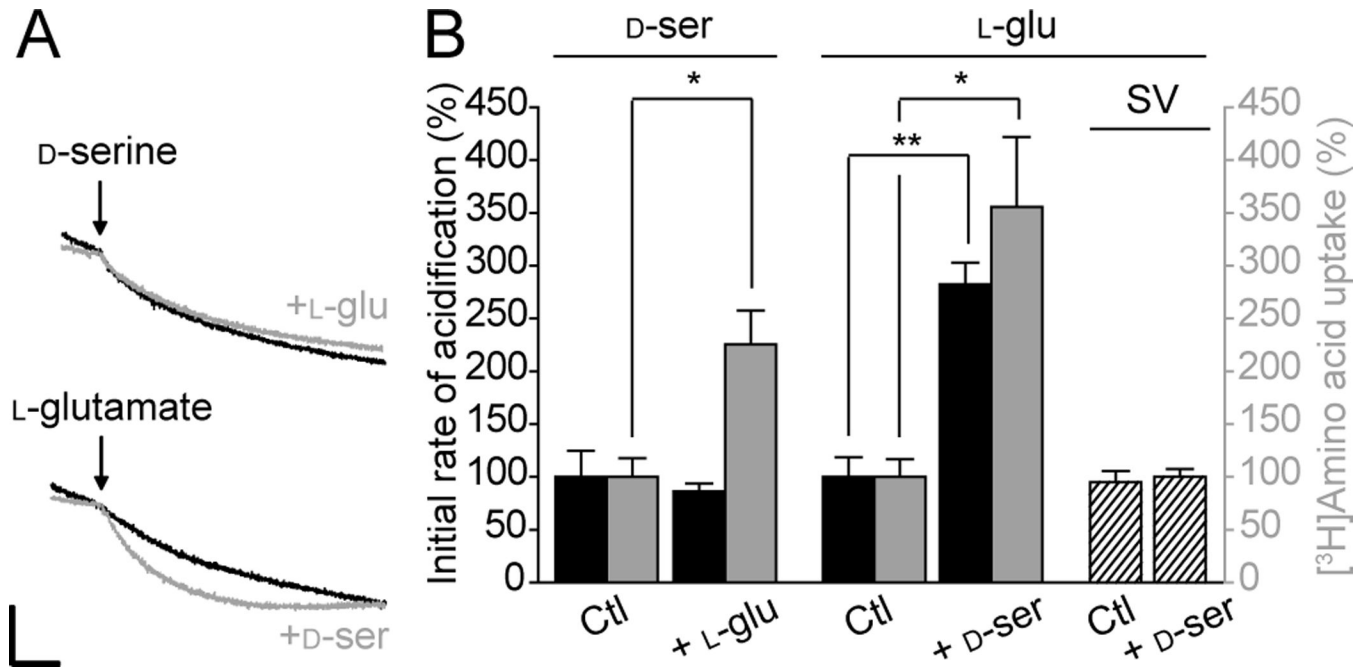
Figure 9.
Vesicular synergy between D-serine and L-glutamate transport activities in GVs. *A*, Partial photometric traces showing the acidification induced by 5 mM D-serine (upper part) and 5 mM L-glutamate (lower part) in absence (black) or presence (grey) of 5 mM L-glutamate and 5 mM D-serine, respectively. The experiments were performed as in Fig. 7, i.e. initiated with 10 mM ATP and ended with 50 mM $(NH_4)_2SO_4$. Scale bar represents 2 a.u. per 1 min. *B*, Histogram showing the reciprocal effect of each amino acid (D-serine or L-glutamate) on the acidification rate and uptake of the other one. Amino acid-induced acidification was measured as in panel *A*. FCCP-sensitive ATP-dependent uptakes of L-[^{3}H]glutamate or D-[^{3}H]serine were measured in presence of 10 mM D-serine or L-glutamate, respectively. Error bars represent mean ± SEM; n = 3 for acidification, 4 in duplicate for radioactive assay. *P<0.05, **P<0.01, Student's t test.
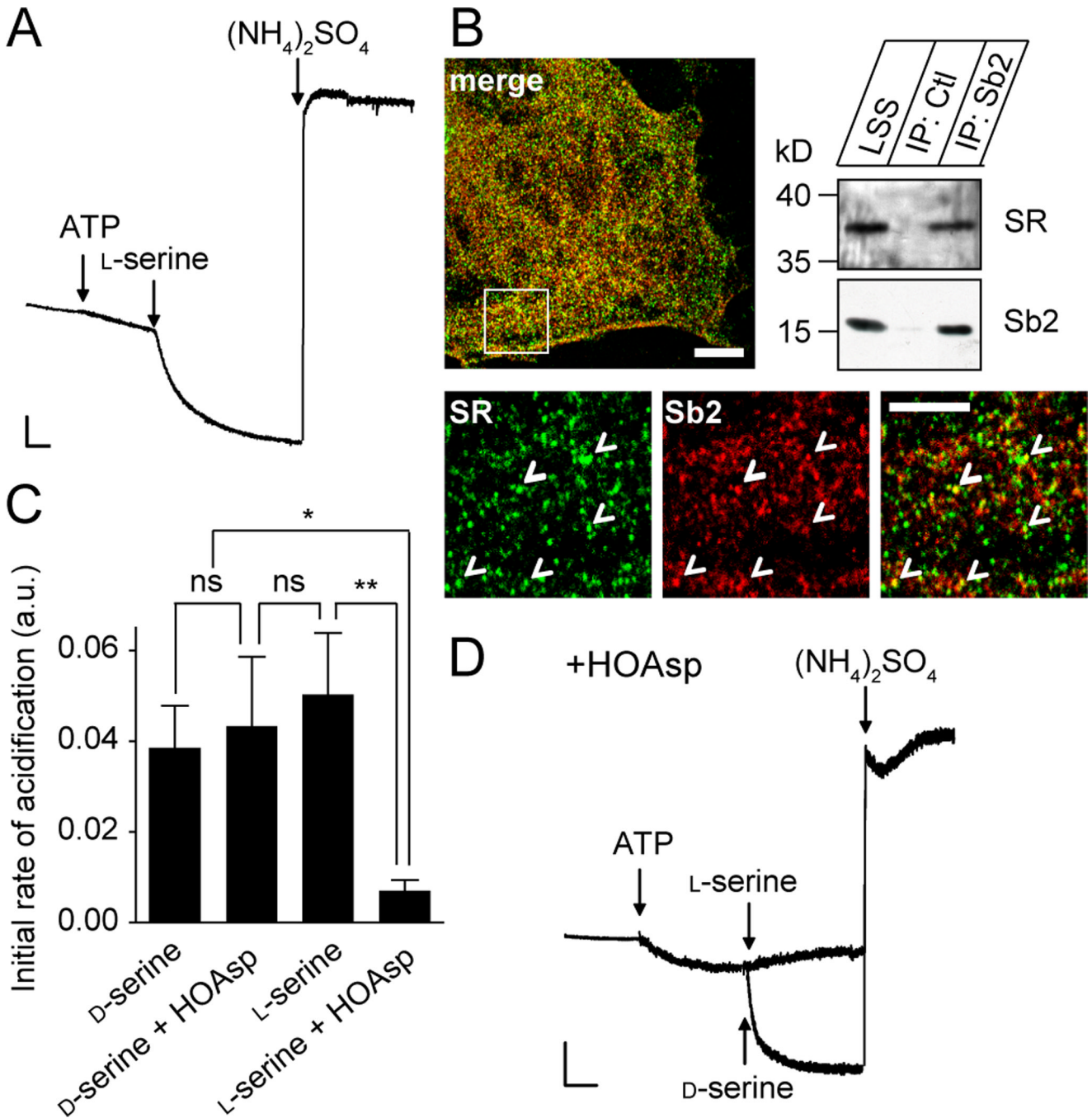
Figure 10.
D-serine uptake by immunoisolated GVs is coupled to endogenous serine racemase activity. **A**, Photometric trace showing acidification induced by 10 mM L-serine (representative trace from 4 independent experiments). Scale bar: 2 a.u. per 1 min. **B**, Colocalization of serine racemase (SR, green) with synaptobrevin 2 (Sb2, red) in cultured astrocytes. Scale bars: 10 μm for the upper image (low magnification) and 5 μm for the zooms. The presence of serine racemase on GVs is confirmed by immunoblotting of immunoisolated GV fractions. **C**, **D**, Inhibition of serine racemase by L-erythro-3-hydroxyaspartate (HOAsp, 400 μM, 10 min) abolishes L-serine-induced acidification without affecting D-serine-induced acidification (10

mM each, representative traces from 4–5 independent experiments). Scale bars: 2 a.u. per 1 min. ns: not significant, *P<0.05, **P<0.01, Student's t test.
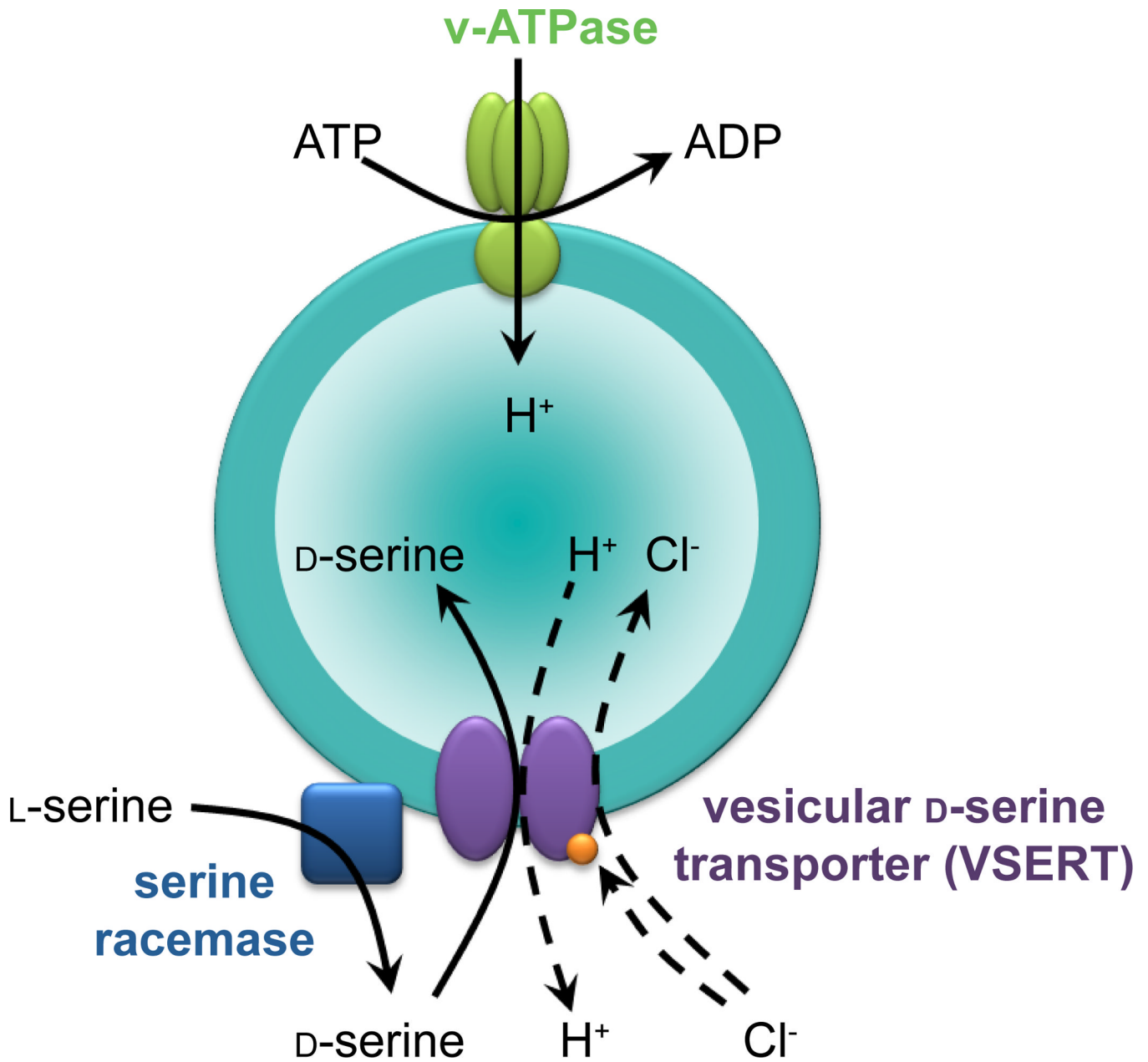
Figure 11.
Vesicular D-serine transporter (VSERT) operates as a putative D-serine/H^+ antiporter with a chloride leak or a coupled chloride cotransport. While D-serine is a neutral amino acid, chloride acts as a counter-ion for V-ATPase and increases the pH gradient while reducing the membrane potential. Alternatively, chloride could modulate D-serine transport by regulatory binding to the transporter. Serine racemase is anchored to the membrane of GVs where it converts L-serine into D-serine allowing a local and efficient concentration of the gliotransmitter to be transported. However, pre-existing D-serine is also taken up in GVs without any preference over a newly synthesized one.