Gliotransmission: focus on exocytotic release of L-glutamate and D-serine from astrocytes

Magalie Martineau*1

*Department of Cellular Biophysics, Institute for Medical Physics and Biophysics, University of Münster, 48149 Münster, Germany

Abstract
The release of neuromodulators, called gliotransmitters, by astrocytes is proposed to modulate neurotransmission and synaptic plasticity, and thereby cognitive functions; but they are also proposed to have a role in diverse neurological disorders. Two main routes have been proposed to ensure gliotransmitter release: non-exocytotic release from cytosolic pools through plasma membrane proteins, and Ca2+-regulated exocytosis through the fusion of gliotransmitter-storing secretory organelles. Regulated Ca2+-dependent glial exocytosis has received much attention and is appealing since its existence endows astrocytes with some of the basic properties thought to be exclusive to neurons and neuroendocrine cells. The present review summarizes recent findings regarding the exocytotic mechanisms underlying the release of two excitatory amino acids, L-glutamate and D-serine.

Introduction
Since the early 1990s, the role of astrocytes as active partners influencing synaptic transmission and plasticity has received considerable attention. This new area of research is substantiated by the concept of tripartite synapse where astrocytic processes are in tight structural and functional relationship with the neuronal pre- and post-synaptic elements [1]. This concept is based on the observation that activation of astrocytes by neurotransmitters triggers the release of gliotransmitters which can directly regulate synapse activity. Among gliotransmitters, L-glutamate and D-serine emerge as important neuroinformative molecules with potential relevance for cognitive functions and brain disorders [1]. Consistent with its role as the main excitatory signalling molecule, L-glutamate released from astrocytes was shown to increase neuronal excitability and potentiate synaptic transmission (see [2] for a detailed review on the functional role of gliotransmission). Also, glial D-serine, the endogenous co-agonist of synaptic NMDA (N-methyl-D-aspartate) receptors, was shown to contribute to activity-induced synaptic plasticity in many areas of the brain [2]. In addition, both L-glutamate and D-serine are involved in NMDA receptor-mediated neurotoxicity [3,4]. Astrocytes are capable of releasing gliotransmitters using two main routes: from cytosolic pools through plasma membrane channels and transporters, and through Ca2+-dependent exocytosis [5]. It is likely that each mechanism is recruited during different physiopathological states of the brain. In the present review, I focus on the exocytotic release of L-glutamate and D-serine, which have aroused increasing interest, but intense controversy.

Gliotransmitter release
The release of L-glutamate was detected in cultured astrocytes using ‘sniffer’ cells expressing glutamatergic receptors [6,7], a fluorescent enzymatic assay [8–11] or by amperometry to detect the release of dopamine as a ‘surrogate’ transmitter [12], whereas the release of D-serine was first characterized in vitro using a luminescent enzymatic assay [13] followed by in vivo measurements by microdialysis [14,15] or more recently by means of amperometric biosensors [16,17]. An increase in cytosolic Ca2+ is necessary and sufficient to induce both L-glutamate and D-serine release from astrocytes. Indeed, application of a Ca2+ ionophore to astrocytes triggered the release of L-glutamate and D-serine in the presence of external free Ca2+ [8,11–13]. Accordingly, stimulation that increased cytosolic Ca2+, including mechanical stimulation [7–9,12,18], photolysis of caged Ca2+ [19,20] and activation of G-protein-coupled or ionotropic receptors [6,10–12], triggered release of L-glutamate. Release of D-serine is driven by activation of glutamatergic and bradykininergic receptors which also caused intracellular Ca2+ increase [13,21]. The precise nature of glutamatergic receptors has not been defined and should be carefully considered [22]. The contribution of cytosolic Ca2+ was further confirmed by application of thapsigargin, a blocker of store-specific Ca2+-ATPase, or by buffering cytosolic Ca2+ with BAPTA [1,2-bis-(o-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid],

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Abbreviations used: ER, endoplasmic reticulum; GABA, γ-aminobutyric acid; NMDA, N-methyl-D-aspartate; α2δ,imately associated protein; SNAP, soluble N-ethylmaleimide-sensitive fusion protein; V-ATPase, vacuolar-type H+-ATPase; VGLUT, vesicular glutamate transporter.

*email magmart@uni-muenster.de
which inhibited both L-glutamate and D-serine release in cultured astrocytes [8–11,13,19], but also in ex vivo preparations [23–26] or in vivo [25]. As seen so far, the Ca\(^{2+}\) necessary for gliotransmitter release seems to originate principally from the ER (endoplasmic reticulum) through IP\(_{3}\) [inositol 1,4,5-trisphosphate]- and ryanodine-operating channels [8,14,23], but mitochondria could be also involved in the Ca\(^{2+}\) signalling necessary for gliotransmission through the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger [27] or cyclophilin D [28]. Precise electron and fluorescence microscopy analyses reported that ER was found beneath astrocytic plasma membrane, in close proximity to vesicles [29,30].

Astrocytes thus possess functional nanodomains where the Ca\(^{2+}\) increase occurred in spatial and temporal correlation with vesicular fusion events [29]. Interestingly, recent evidence demonstrates that TRP (transient receptor potential) channel-mediated transmembrane Ca\(^{2+}\) influx may also trigger astrocytic L-glutamate [31] or D-serine release [17], as well as the plasma membrane Na\(^{+}\)/Ca\(^{2+}\) exchanger releasing L-glutamate while operating in the reverse mode [32]. Thus different intracellular endomembranes (ER and mitochondria) together with plasma membrane exchangers, transporters, channels and receptors seem to cross-talk in a complex interplay to drive gliotransmission. Complexity is added considering that Na\(^{+}\) in addition to Ca\(^{2+}\) might also play an important role [27,32].

Ca\(^{2+}\)-dependent release of L-glutamate and D-serine depends on SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) proteins which mediate fusion between vesicular and plasma membranes. Cleavage of the vesicular SNARE proteins Sbh2 (synaptoprevin 2) and cellubrevin by tetanus toxin or botulinum toxin B caused a strong inhibition of Ca\(^{2+}\)-dependent L-glutamate and D-serine release [6–9,13,18,26]. The expression of the SNARE motif of Sbh2 (also referred to as dominant-negative SNARE) which prevents exocytosis reduced L-glutamate release from astrocytes [11]. The possible release of gliotransmitters by exocytosis is appealing, but raises the question of the Ca\(^{2+}\) sensor which triggers regulated exocytosis in astrocytes. Interestingly, reduction of the Ca\(^{2+}\)-binding vesicular protein synaptotagmin 4 in astrocytes by RNA interference or mutation of the Ca\(^{2+}\)-binding domain in synaptotagmin 4 decreased Ca\(^{2+}\)-dependent L-glutamate release [10]. The effect of such genetic manipulations on D-serine release has not so far been investigated. In synaptic vesicles, the concentration of neurotransmitters against their gradient is fuelled by a proton electrochemical gradient generated by a V-ATPase (vacuolar-type H\(^{+}\)-ATPase) [33]. Blockade of V-ATPase with bafilomycin A1 or concanamycin A inhibited L-glutamate and D-serine release from astrocytes [8,9,11,13,18], indicating that astrocytic vesicles may use the same mechanism as synaptic vesicles to package gliotransmitters.

Glutamate and D-serine undergo Ca\(^{2+}\)-dependent and SNARE protein-dependent release, suggesting that astrocytes can exocytose gliotransmitters. Nowadays, evidence is mostly indirect and the exocytosis hypothesis is weakened by the observation that astrocytes can also release gliotransmitters directly from the cytosol [5]. Therefore the vesicular mode of gliotransmitter release is actively debated [22,34].

The possibility cannot be excluded that gliotransmitters are released through non-vesicular mechanisms, whereas exocytosis is required to address receptors and transporters at the plasma membrane or to release a ‘trigger molecule’ which is responsible for non-exocytotic gliotransmitter release. Taking these facts into consideration, the concept of Ca\(^{2+}\)-regulated gliotransmitter release still awaits direct evidence. A first step for the validation of the concept of gliotransmitter exocytosis is to demonstrate the presence of and characterize the endomembrane compartments that store gliotransmitters, to define their mode of fusion with the plasma membrane, and to demonstrate accumulation of gliotransmitter inside organelles.

**Gliarial vesicles: storage carriers for gliotransmitters**

Secretory vesicles are the fundamental morphological elements for regulated Ca\(^{2+}\)-dependent exocytosis. On the basis of electron microscopy, astrocytes possess electron-lucent (clear) vesicular structures with a mean diameter of 30–50 nm. In hippocampal astrocytes in situ, VGLUT1 (VGLUT is vesicular glutamate transporter) and 2 are associated with small clear vesicles with a mean diameter of ~30 nm [6]. Glutamate and D-serine accumulate in clear vesicles with a diameter of ~36 nm in the perisynaptic processes of hippocampal and cortical astrocytes [30,35]. In addition, clear vesicles observed in gliosomes (pinched-off astrocytic processes) measure ~30 nm in diameter [36], whereas immunolocalized Sbh2-containing vesicles from cultured cortical astrocytes measure ~40 nm [35], and recycling vesicles which captured antibodies against VGLUT1 have a diameter of ~50 nm [37]. In the adult hippocampus, clear vesicles are organized in small clusters of 2–15 vesicles preferentially located within 100 nm of the astrocytic plasma membrane [30]. These small clusters are observed at sites adjacent to neuronal elements bearing NMDA receptors [6]. Thus astrocytes possess small vesicles resembling those at synaptic termini [38]; however, at a lower density. Much larger vesicles of 1–3 \(\mu\)m in diameter were shown to release D-serine and L-glutamate. They are formed during sustained high astrocytic Ca\(^{2+}\) concentrations, after weak mechanical stimulation or after repetitive local application of high L-glutamate concentrations [39,40], questioning the role of these large vesicles in physiological processes. They are larger than the diameter of most astrocytic processes [30] and so might form only in reactive astrocytes under pathological conditions. Finally, even if lysosomes were shown to contribute to Ca\(^{2+}\)-dependent exocytosis, they are not responsible for the release of L-glutamate and D-serine [7,21].

Fusion of vesicles with the plasma membrane is promoted by the formation of the SNARE complex which spans between the vesicular and plasma membranes [41]. At
neuronal synapses, Sb2 present in the vesicular membrane forms a SNARE complex with SNAP25 (synaptosome-associated protein 25) and syntaxin 1 present at the plasma membrane. The complex formation is regulated by Ca\(^{2+}\) which binds to vesicular synaptotagmin 1 leading to the fusion of this protein with the plasma membrane, thus initiating fusion [41]. The astrocytic analogues of these proteins are Sb2 and its homologue cellubrevin, SNAP23 and syntaxin 1, as well as the auxiliary protein synaptotagmin 4 (reviewed in [42]). Astrogial vesicles also possess proteins necessary for vesicular filling, such as the V-ATPase [35,43], which provides the proton driving force to fuel transmitter loading, and all three isoforms of VGLUT ([6,9,11,35,44,45], but see [46]). VGLUT is active at the vesicular membrane of astrocytes since its inhibition by Evans Blue, Rose Bengal or Trypan Blue led to the reduction in evoked \(\text{L-glutamate}\) release [7,9,26]. Although the vesicular transporter for D-serine has not been identified, the transport of D-serine inside fusion type on gliotransmitter release and, most important, on fusion, the relative amount of the two types of fusion is spontaneous events have an equal ratio of full to transient a fusion pore without collapsing [12,29,48,49]. Although spontaneous events have an equal ratio of full to transient fusion, the relative amount of the two types of fusion is dependent on the stimulus applied [48,49]. The role of each fusion type on gliotransmitter release and, most important, on astrocytic modulation of neuronal activity, requires further investigation.

### Vesicular transport of gliotransmitters

Astrocytes possess secretory vesicles equipped with the proteins necessary to undergo exocytosis. In addition, immunogold electron microscopy on the adult hippocampus and cerebral cortex, as well as capillary electrophoresis on isolated Sb2-containing vesicles showed that small clear astrocytic vesicles store both gliotransmitters, \(\text{L-glutamate}\) and D-serine [30,35]. The vesicular concentration of fixed gliotransmitters estimated by immunogold cytochemistry is in the millimolar range, comparable with the concentration of L-glutamate in synaptic vesicles [30]. Since \(\text{L-glutamate}\) and D-serine are synthesized in the cytoplasm of astrocytes, their exocytotic release requires their transport from the cytoplasm into the astrocytic vesicles.

Vesicular transmitter transport was extensively characterized on synaptic vesicles, dramatically contrasting with only a few studies addressing the features of \(\text{L-glutamate}\) and D-serine transport into astrocytic vesicles. Similar to synaptic vesicles, vesicular transport of both gliotransmitters is dependent on a proton electrochemical gradient established by V-ATPase [35,45]. The proton electrochemical gradient has two components: the membrane potential and the pH gradient. In synaptic vesicles, the relative contribution of the two components is determined by the presence of chloride, which acts as a counterion for protons and thus acidifies the vesicular lumen [33]. No acidification was observed when chloride was added to isolated astrocytic vesicles [35]. In contrast with synaptic vesicles, astrocytic vesicles appeared intriguingly impermeant to chloride. However, extravesicular chloride modulates both \(\text{L-glutamate}\) and D-serine transport into astrocytic vesicles, reaching a maximum activity at 4 mM [35]. The apparent affinity of \(\text{L-glutamate}\) transport is \(\sim 2\) mM, consistent with the affinity of VGLUT for \(\text{L-glutamate}\) in synaptic vesicles or proteoliposomes [50,51]. The role of chloride in vesicular glutamate transport is still a matter of debate. Chloride was proposed to be co-transported with \(\text{L-glutamate}\) and/or to act as an allosteric modulator of VGLUT activity [51,52]. The selective dissipation of either one or the other component of the proton electrochemical gradient indicated that VGLUT transports \(\text{L-glutamate}\) in synaptic vesicles mostly depending on the membrane potential [33]. This characteristic was not yet confirmed in astrocytic vesicles. In addition, vesicular \(\text{L-glutamate}\) content is dependent on VGLUT3 copy number and the cytosolic gliotransmitter concentration. Indeed, Ni and Parpura [53] showed that overexpression of VGLUT3 (but not VGLUT1 or VGLUT2) or an increased cytosolic \(\text{L-glutamate}\) concentration enhanced \(\text{Ca}^{2+}\)-dependent \(\text{L-glutamate}\) release from astrocytes. Although \(\text{L-glutamate}\) transport was observed in both synaptic and astrocytic vesicles, the transport of D-serine is specific to astrocytic vesicles [35]. Its apparent affinity is \(\sim 7\) mM, consistent with the affinity of VIAAT (vesicular inhibitory amino
Acid transporter) for GABA (γ-aminobutyric acid), another neutral amino acid [33,35]. Similar to GABA, the transport of D-serine in astrocytic vesicles was shown to rely evenly on both components of the proton electrochemical gradient [33,35]. Because D-serine transport induced vesicular acidification and is completely dependent on the presence of chloride, the vesicular D-serine transporter was proposed to be a D-serine/chloride co-transporter [35]. However, the molecular identification of the vesicular D-serine transporter requires further investigation. A spatial association of serine racemase activity and D-serine vesicular transport was observed, resulting in a functional coupling between D-serine synthesis and uptake [35]. Even though this coupling has been demonstrated for L-glutamate in synaptic vesicles [54], it has not yet been addressed in astrocytic vesicles. Finally, D-serine and L-glutamate vesicular loading exert a mutual stimulation which indicates a functional cross-talk between the two transporters [35]. This vesicular synergy can only be explained by both transporters residing on the same vesicle, indicating the co-storage and thus the co-release of both gliotransmitters. The immunogold co-labelling of D-serine and L-glutamate in the adult hippocampus did not reveal a population of vesicles containing both gliotransmitters, probably due to the limited sensitivity of the technique [30]. The mechanism underlying the vesicular synergy between D-serine and L-glutamate uptake requires additional investigation. It may reflect their common dependency on chloride or their relative relationship with both components of the proton electrochemical gradient. Nevertheless, the possible co-storage of L-glutamate and D-serine, at least in a subpopulation of glial vesicles, raises the interdependence of their dynamics and functions at regulating synaptic and extrasynaptic functions.

Conclusion and perspective

The tripartite synapse concept assumes that astrocytes integrate the level of synaptic activity and in turn influence its efficacy through the regulated release of gliotransmitters [1]. The ability of astrocytes to sense neuronal activity and reply to it was analysed mostly on cell culture systems, which resulted in a simplified model for neuron–glia communications. It is of paramount importance now to go beyond the cellular mechanisms of gliotransmission and establish the functional relevance of gliotransmitter release in normal brain processing. The vast majority of studies which address this issue on brain slices or in vivo involve the recording of neuronal electrical activity as a readout for synaptic activity [55]. In addition, methods for activating or blocking astrocytes appear too non-specific and tools are lacking that selectively have an impact on astrocyte function without simultaneously affecting other cells, notably neurons (reviewed in [34]). Therefore the development of new readouts directly addressing astrocytes and improved tools to selectively control astrocyte activity in vivo may help to fill the gap between understanding gliotransmission in a dish and unravelling astrocytic modulation of synaptic activity in an integrated neuron–glia network. Ultimately, moving towards a more physiological model will provide evidence as to whether gliotransmitter exocytosis occurs in vivo and how it is regulated.

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