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Gliotransmission: Exocytotic release from astrocytes

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Abstract

Gliotransmitters are chemicals released from glial cells fulfilling a following set of criteria: i) they are synthesized by and/or stored in glia; ii) their regulated release is triggered by physiological and/or pathological stimuli; iii) they activate rapid (milliseconds to seconds) responses in neighboring cells; and iv) they play a role in (patho)physiological processes. Astrocytes can release a variety of gliotransmitters into the extracellular space using several different mechanisms. In this review, we focus on exocytotic mechanism(s) underlying the release of three classes of gliotransmitters: (i) amino acids, such as, glutamate and D-serine; (ii) nucleotides, like adenosine 5'-triphosphate; and (iii) peptides, such as, atrial natriuretic peptide and brain-derived neurotrophic factor. It is becoming clear that astrocytes are endowed with elements that qualify them as cells communicating with neurons and other cells within the central nervous system by employing regulated exocytosis.

Keywords

astrocytes; exocytosis; glutamate; D-serine; ATP; atrial natriuretic peptide; brain-derived neurotrophic factor

Introduction

The criteria for a chemical released from neurons to be classified as a neurotransmitter have been defined and frequently modified [6,15]. Since transmitter release from glia was demonstrated at a much latter time than that from neurons [16], only recently has a similar set of criteria been put forth [27,52,91] to establish what compounds qualify as “gliotransmitters”: i) synthesis by and/or storage in glia; ii) regulated release triggered by physiological and/or pathological stimuli; iii) activation of rapid (milliseconds to seconds) responses in neighboring cells; and iv) a role in (patho)physiological processes.

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Astrocytes and other glial cells can release a variety of transmitters into the extracellular space using several different mechanisms: (i) through channels like anion channel opening, induced by cell swelling [67], release through functional unpaired connexons/pannexons, “hemichannels”, on the cell surface [23,39] and ionotropic purinergic receptors [29]; (ii) through transporters, such as, reversal of uptake by plasma membrane excitatory amino acid transporters [87], exchange via the cystine-glutamate antiporter [93] or organic anion transporters [77]; and (iii) through Ca^{2+}-dependent exocytosis [62].

In this review we focus on the exocytotic mechanism(s) underlying the release of three classes of gliotransmitters: (i) amino acids, such as glutamate and D-serine; (ii) nucleotides, like adenosine 5'-triphosphate (ATP); and (iii) peptides, such as, atrial natriuretic peptide (ANP) and brain-derived neurotrophic factor (BDNF). We only disclose the consequences of transmitter release from astrocytes onto neighboring cells when the effect of transmitter release from astrocytes is used as an assay for release.

Amino acids as astrocytic transmitters

Glutamate is synthesized within astrocytes as a by-product of the tricarboxylic acid (TCA) cycle. Since astrocytes possess the enzyme pyruvate carboxylase, they can synthesize glutamate *de novo* [35]. Glutamate is converted from the TCA cycle intermediate, α-ketoglutarate, usually via transamination of another amino acid, such as, aspartate [94](Figure 1). D-serine is converted from L-serine by the action of serine racemase, an enzyme found predominately in astrocytes [97].

Evidence for Ca^{2+}-dependent glutamate release from astrocytes was first shown using high performance liquid chromatography to monitor the release of this transmitter from cultured astrocytes [62]. Astrocytes were equilibrated for prolonged periods of time (40–60 minutes) either in a solution containing normal external free Ca^{2+} (2.4 mM), or in a solution depleted of external free Ca^{2+} (24 nM); the latter solution caused a depletion of internal Ca^{2+} stores and prevented Ca^{2+} entry from the extracellular space. Addition of the Ca^{2+} ionophore, ionomycin, in the presence of normal external Ca^{2+}, caused an increase in the release of glutamate from astrocytes. Stimulation of astrocytes, bathed in a solution depleted of free Ca^{2+}, failed to cause an increase in glutamate release. These data indicate that elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is sufficient and necessary to stimulate glutamate release. Consistent with the former finding, other stimuli that directly increased astrocytic $[Ca^{2+}]_i$, including mechanical stimulation [4,5,36,57,62], photostimulation [62], and photolysis of Ca^{2+} cages [5,64,100], all caused release of glutamate. The notion that elevated $[Ca^{2+}]_i$ is necessary for glutamate release from astrocytes is further supported by the reduction of the evoked glutamate release from astrocytes when the astrocytic buffering capacity for cytosolic Ca^{2+} was augmented using the Ca^{2+} chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), or when $[Ca^{2+}]_i$ increases where dampened by the depletion of internal Ca^{2+} stores due to pre-incubation of these cells with thapsigargin, a blocker of store specific Ca^{2+}-ATPase [4,12,36,40,57].

The majority of intracellular Ca^{2+} necessary for glutamate release originates from endoplasmic reticulum (ER) internal stores (Figure 2), since Ca^{2+}-dependent glutamate release from astrocytes is most prominently reduced in the presence of thapsigargin [36]. Diphenylboric acid 2-aminoethyl ester (2-APB) solution, a cell-permeant inositol 1,4,5-trisphosphate (IP_3) receptor antagonist, greatly reduced exocytotic glutamate release from astrocytes, implicating the role of IP_3- sensitive internal stores in mediating Ca^{2+}-dependent glutamate release from astrocytes. Similarly, ryanodine/caffeine-sensitive ER stores play a role, as well, since the treatment of astrocytes with ryanodine, at concentrations that blocked the release of Ca^{2+} from the ryanodine/caffeine- sensitive stores, also attenuated mechanically-induced glutamate

release. Furthermore, the sustained presence of caffeine, that depleted ryanodine/caffeine stores, also reduced mechanically-induced glutamate release. Thus, Ca^{2+}-dependent glutamate release from astrocytes requires co-activation of IP_3- and ryanodine/caffeine-sensitive internal Ca^{2+} stores, which operate jointly [36]. It should be noted, however, that the functionality of ryanodine receptors in astrocytes is still debated, since the lack of their activity in astrocytes *in situ* had been reported [9]. Nonetheless, increase in Ca^{2+} within subplama membrane cytosolic microdomains, delimited by ER and containing glutamatergic vesicles, controls exocytotic vesicular fusions [51].

Ca^{2+} entry from the extracellular space across the astrocytic plasma membrane is ultimately required for the (re)filling of ER Ca^{2+} stores (Figure 2). This occurs via so-called store-operated Ca^{2+} (SOC) channels [33,88]. Astrocytes express canonical transient receptor potential (TRPC) channels, implicated in SOC entry, which play a role in the regulation of Ca^{2+} homeostasis in these cells [33,34,69]. Specifically, TRPC1 functionally contributes to Ca^{2+}-dependent glutamate release from astrocytes [50], since an antibody against TRPC1, that was designed to bind to the pore forming region of TRPC1 and that blocks the functioning of the channel [92], caused a significant decrease in the measured SOC entry and mechanically-induced glutamate release from these cells. This is consistent with the finding that the presence of extracellular Cd^{2+}, a blocker of Ca^{2+} entry from the extracellular space, reduces mechanically-induced Ca^{2+}-dependent glutamate release from astrocytes [36]. Voltage-gated Ca^{2+} channels (Parri et al., 2001) might mediate additional entry of Ca^{2+} from the extracellular space that is utilized to trigger exocytotic glutamate release from astrocytes of ventrobasal thalamus. The role of the ionotropic transmitter receptors, which represent an additional entry of Ca^{2+} in astrocytes [reviewed in [90]], in exocytotic glutamate release from astrocytes is intangible at the moment [reviewed in [75]].

Mitochondria can modulate intracellular Ca^{2+} handling and affect exocytosis in astrocytes [74]. These organelles possess a mitochondrial Ca^{2+} uniporter that can transport Ca^{2+} into the mitochondrial matrix and it operates at cytosolic $[Ca^{2+}]$ greater than ~ 0.5 μM [55,80]. Blocking this uniporter with ruthenium 360 increased mechanically-induced cytosolic Ca^{2+} accumulation and glutamate release in cortical astrocytes. Conversely, decreasing mitochondrial Ca^{2+} efflux by blocking the mitochondrial Na^+/Ca^{2+} exchanger with 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), or increasing mitochondrial Ca^{2+} load by inhibiting formation of the mitochondrial permeability transition pore with cyclosporin A, decreased cytosolic Ca^{2+} accumulation and glutamate release in cortical astrocytes. Taken together, these data suggest that mitochondria have the capacity to modulate the magnitude of Ca^{2+}-dependent glutamate release from astrocytes (Figure 2).

Ca^{2+}-dependent release of transmitters depends on the presence of exocytotic secretory machinery. Indeed, astrocytes express the proteins of the soluble N-ethyl maleimide-sensitive fusion protein attachment protein receptor (SNARE) complex: synaptobrevin 2 [(Sb2), also referred to as vesicle–associated membrane protein 2 (VAMP 2)], syntaxin 1, synaptosome-associated protein of 23 kDa (SNAP-23), as well as several ancillary proteins to this complex, including synaptotagmin 4 [reviewed in [56]] (Figure 1). The use of *Clostridial*, tetanus, and various types of botulium toxins, which cleave SNARE proteins necessary for exocytosis, caused a reduction in the level of Ca^{2+}-dependent glutamate release in astrocytes [reviewed in [56]]. Additionally, the use of tetanus toxin, which cleaves astrocytic Sb2 and cellubrevin [63], caused a reduction in plasma membrane capacitance (C_m) increase [46], and a reduction in the number of amperometric spikes [21], both reporting on exocytosis from astrocytes. The experimental manipulation of the SNARE complex by expressing the cytoplasmic domain of Sb2, that acted as a dominant negative SNARE, resulted in the inhibition of glutamate release from astrocytes [100]. It should be noted that Sb2 cytosolic domain contains the SNARE domain, but lacks the ability to anchor to the vesicular membrane. Similarly, the expression of

mutated synaptotagmin 4, acting in a dominant-negative manner, caused the reduction of Ca^{2+}-dependent glutamate release from astrocytes [99]. Furthermore, α-latrotoxin, an active component of black widow spider venom, which binds to neurexins/latrophilins to induce release of transmitter by stimulating the secretory machinery [reviewed in [86]], has been shown to cause glutamate release from astrocytes [42,65].

Proteins utilized for sequestering glutamate into vesicles have also been found in astrocytes (Figure 1). Hence, the vacuolar type of proton ATPase (V-ATPase), which drives protons into the vesicular lumen creating the proton concentration gradient necessary for glutamate transport into vesicles, has been detected in astrocytes [95]. Its blockage with bafilomycin A_1 reduces glutamate release from astrocytes caused by various stimuli [3,13,24,57,68]. The three known isoforms of vesicular glutamate transporters (VGLUTs) 1, 2 and 3, which use the proton gradient created by V-ATPases to package glutamate into vesicles, have been detected in astrocytes [2,14,24,32,46,57,100]. These transporters are functional within astrocytes since Rose Bengal, an allosteric modulator of VGLUTs, greatly reduced glutamate release [57]. VGLUT3 and the cytosolic concentration of glutamate appear to be key limiting factors in regulating the Ca^{2+}-dependent release of glutamate from astrocytes [60] (Figure 1). Selective over-expression of individual VGLUT proteins in astrocytes showed that VGLUT3, but neither VGLUT1 nor VGLUT2, enhanced mechanically-induced Ca^{2+}-dependent glutamate release. Similarly, inhibition of glutamine synthetase activity by L-methionine sulfoximine in astrocytes, which leads to increased cytosolic glutamate concentration, greatly increased their mechanically-induced Ca^{2+}-dependent glutamate release without affecting intracellular Ca^{2+} dynamics [60].

Secretory vesicles are the essential morphological elements for regulated Ca^{2+}-dependent exocytosis. Hence, based on immunoelectron microscopy (IEM), Sb2 can be found located in vicinity of electron-lucent (clear) vesicular structures [49], while VGLUTs 1 or 2 in astrocytes *in situ* were found associated with small, clear vesicles with a mean diameter of ~30 nm [14]. Similarly, immunoisolated Sb2-containing vesicles that originated from cultured astrocytes [24] were heterogeneous in size, ranging from 30 to over 100 nm, and predominately appeared as electron-lucent. Recycling glutamatergic vesicles which can capture the extracellular antibody against VGLUT1 in a Ca^{2+}-dependent manner are electron-lucent and have a diameter of ~ 50 nm [83]. Furthermore, gliosomes [85], pinched off astrocytic processes, expressing Sb2 and VGLUT 1, contained clear vesicles with diameters of ~30 nm; some of them were clathrin-coated vesicles. Much larger vesicles, over 1 μm in diameter, have been observed to form within minutes of repeated stimulation with pharmacological dosages (5–50 mM) of glutamate [44,98]; these vesicles can release glutamate, although it is highly likely that they represent a pharmacologically-induced phenomenon or may play a role in pathological processes [see also the discussion in [11]]

The recycling of secretory vesicles at the plasma membrane has been investigated in astrocytes using fluorescence microscopy. Application of ionomycin in the presence of extracellular Ca^{2+}, but not in its absence, caused uptake of the membrane recycling dye, FM 4–64 [47]. Similarly, using a pre-loading technique that stimulated membrane recycling and the trapping of styryl dyes (FM 1–43 or FM 2–10) in secretory organelles, astrocytes displayed a punctate pattern of FM fluorescence [21]. Trafficking of glutamatergic vesicles in astrocytes was assessed using an immunological approach. Hence, after increasing cytoplasmic Ca^{2+} levels in astrocytes in the presence of antibodies against VGLUT1 in extracellular space, presumably binding to luminal/intravesicluar epitope of this transporter, there was an increase in intracellular fluorescent puncta [83]. The delivery of secretory vesicles and fusion to the plasma membrane was also studied in astrocytes. Crippa et al. [24] expressed a chimeric protein, where enhanced green fluorescent protein (EGFP) was fused to the C-terminus of Sb2 (Sb2-EGFP), in astrocytes. Since the C-termimus of Sb2 is located in the vesicular lumen, EGFP was targeted

intravesicularly. When astrocytes were stimulated with Ca^{2+} ionophore, many fluorescent Sb2-EGFP puncta vanished with a simultaneous increase in plasma membrane fluorescence, consistent with regulated exocytosis and fusion of labeled vesicles to the plasma membrane. Net addition of vesicular membrane to the plasma membrane can be directly measured by monitoring changes in C_m. Indeed, an agonist-induced rise in astrocytic $[Ca^{2+}]_i$, causing regulated exocytosis, resulted in an increase of C_m, while concomitant measurements recorded a release of glutamate [100]. Further evidence for vesicular exocytosis from astrocytes was provided by total internal reflection fluorescence (TIRF) microscopy [14,17,28,51], where exocytosis of VGLUT1-,VGLUT2- or Sb2- positive vesicles were reported. As a consequence of vesicular fusions, quantal events of glutamate release, representing an exocytotic hallmark [26], have been recorded from astrocytes. Such events were detected using reporter cells expressing N-methyl-D-aspartate (NMDA) receptors [68], or by amperometric measurements used to detect the release of dopamine, acting as a “surrogate” transmitter for glutamate, from glutamatergic vesicles [21].

Astrocytes can also release the amino acid D-serine [79], a ligand to the glycine modulatory binding site of the NMDA receptor. Mothet at al. [58] investigated the mechanism of this release using an enzyme-linked assay to measure extracellular D-serine concentration. Following glutamate receptor stimulation, astrocytes released D-serine in a Ca^{2+}-dependent manner; the release was augmented by Ca^{2+} and inhibited by application of thapsigargin or removal of extracellular Ca^{2+}. Furthermore, this release of D-serine was reduced by concanamycin A, a V-ATPase inhibitor, and tetanus toxin, implicating the involvement of a vesicular mechanism. Consistent with this notion, D-serine was found to co-localize with Sb2 based on immunocytochemistry and fluorescence microscopy. The investigation of the mechanism underlying a Ca^{2+}-dependent release of D-serine from astrocytes was expanded in a subsequent study using confocal fluorescence microscopy [53]. Using pharmacological inhibition of vesicular budding indicated that D-serine was packaged in vesicles down stream of the Golgi apparatus. The molecular identity of the vesicular transporter for D-serine, however, remains undetermined. Nonetheless, the delivery of secretory vesicles and fusion to the plasma membrane showed the recruitment of Sb2 to the plasma membrane with related disappearance of intracellular D-serine punctate stain. Taken together, D-serine appears to be secreted from astrocytes using a regulated exocytosis/vesicular pathway.

ATP as an astrocytic transmitter

ATP is produced via glycolysis and oxidative phosphorylation. Intracellular ATP provides energy for a variety of processes, including vesicular recycling. Once released into extracellular space, ATP can be used in intercellular signaling acting directly onto purinergic receptors. Alternatively, upon its hydrolysis by membrane-bound ecto-nucleotidases, the extracellular degradation products, ADP and adenosine, can activate different plasma membrane receptors [reviewed in [31]]

As already outlined, astrocytes possess secretory vesicles and a variety of exocytotic proteins. Cultured astrocytes investigated under EM displayed large dense core granules with diameters of ~115 nm, containing the secretory peptide secretogranin II [18] and ATP [22]. Based on immunoblotting, subcellular fractions containing secretogranin II were mainly distinct from fractions containing Sb2 [18]. Consistent with this finding, dense core vesicles represented ~2 % of the total number of immuno-isolated Sb2-containing vesicles [24]. Similarly, using IEM, it was demonstrated that Sb2 can be associated with some dense core vesicular structures, with diameters ranging from 100–700 nm [49]. Following subcellular fractionations, immunoblotting for several exocytotic proteins, Sb2, syntaxin 1, cellubrevin and synaptotagmin 1, were found to co-localize with ATP containing organelles [49]. It should be noted, however, that the presence of synaptotagmin 1 was not detected in astrocytes by others

[24,62,99]. The protein responsible for the ATP accumulation in secretroy vesicles has recently been identified as SLC17A9 [78]. This vesicular nucleotide transporter (VNUT) was found present in astrocytes based on immunocytochemistry.

Morphological and biochemical evidence suggests that ATP as an astrocytic transmitter may be released by Ca^{2+}-dependent exocytosis. The first evidence in support of such a notion comes from experiments in which astrocytes exposed to nitric oxide responded with an increase in cytoplasmic Ca^{2+} and the release of ATP to the extracellular space [8]. Buffering of intracellular Ca^{2+} with BAPTA or preventing vesicular release with botulinum toxin C greatly reduced the release of ATP. Furthermore, Coco et al. [22] demonstrated that mechanically stimulated astrocytes released ATP which could be inhibited by application of bafilomycin A_1 or tetanus toxin. Interestingly, the reduction of ATP release caused by tetanus toxin was less pronounced than the reduction in release of glutamate, indicating that ATP and glutamate release may be regulated in a different manner, perhaps through distinct vesicular pools.

ATP release from cultured astrocytes could be evoked by uridine 5'-triphosphate (UTP) via the likely activation of $P2Y_2$ receptors [1]. This release was reduced by thapsigargin and lithium ions that can block the intracellular generation of IP_3. Further pharmacology on vesicular trafficking implicates that the exocytotic pathway is involved in UTP-induced ATP release from astrocytes: a blocker of transport vesicles budding off the Golgi apparatus, brefeldin A, a disruptor of actin microfilaments, cytochalasin D, and the exocytosis inhibitor, botulinum toxin A, all blocked ATP release. However, the pre-incubation with a cell permeable form of BAPTA showed a trend in the reduction of release, although the effect was insignificant; this may be ascribed to an insufficient concentration of BAPTA within the cell.

To study the quantal nature of ATP release from astrocytes, Pangrsic et al. [61] incubated astrocytes with quinacrine, a compound that fluorescently labels ATP containing structures. Using TIRF microscopy, quinacrine showed punctate stain. The rapid loss of these puncta was evident upon receptor stimulation using glutamate or ATP and stimuli that directly raise intracellular Ca^{2+} levels, ionomycin or flash photolysis of caged Ca^{2+} [73]. Expressing a dominant negative SNARE in astrocytes resulted in the inhibition of the Ca^{2+}-induced reduction in the quinacrine fluorescent puncta representing ATP-containing vesicle exocytosis [61]. Glutamate stimulation of astrocytes showed quantal release of ATP as recorded by ATP reporter cells [61], human embryonic kidney cells expressing a mutated $P2X_3$ receptor with reduced desensitization [30].

Under particular experimental conditions, the exocytotic release of ATP stored in astrocytic lysosomes could be detected [101]. Hence, prolonged (more than 1 hour) incubation with FM recycling dyes stained astrocytic lysosomes based on a co-localization of FM and various lysosomal markers under fluorescence microscopy. Agonist stimulation or metabolic blockade of astrocytes revealed regulated exocytosis of these lysosomes under TIRF microscopy that was blocked by intracellular Ca^{2+} buffering with BAPTA. These lysosomes could readily load with the fluorescent ATP analogue MANT-ATP, that was also released upon stimulation. Indeed, the astrocytic lysosomal fraction in density gradient centrifugation contained abundant ATP. Two subsequent studies confirmed that in astrocytes lysosome-like organelles can assume the role of secretory vesicles and undergo Ca^{2+}-depenent exocytosis [41,48]. Thus, it appears that ATP, and perhaps other gliotransmittes, in astrocytes could be stored in at least two distinct organelles, secretroy vesicles and lysosomes, from which it can be released by regulated exocytosis. It will be necessary to determine under which conditions these two distinct pools of organelles would be recruited. For example, do the same organelles deliver transmitter for release under physiological and pathological conditions or are there specific organelles that operate under particular conditions?

Peptides as astrocytic transmitters

In contrast to amino acids and ATP, which are loaded into vesicles by membrane transporters, peptidergic gliotransmitters enter vesicles via the synthetic secretory pathway (Figure 3). Pro-peptides are made in the ER, transit Golgi compartments where they get concentrated and sorted into organelles; then, they are processed to their final form before release [25]. Vesicles carrying peptidergic transmitters appear to have a distinct morphological appearance under EM; they exhibit electron dense cores and are termed dense-core vesicle, large dense-core vesicles or secretory granules. Their diameter is somewhat larger in comparison with the small synaptic-like clear-core vesicles and appear to contain secretogranins [96]. Therefore, in the first studies where the presence of dense-core vesicles in astrocytes was considered, subcellular distribution and the secretory pathway of secretogranin II was studied [18]. The EM results of this study have shown that, in astrocytes, dense-core vesicles are present near the Golgi complex, typically have a diameter of approximately 100 nm, and that secretogranin II is released upon stimulation by different secretagogues, including bradykinin, adenosine 3':5' cyclic monophosphate (cAMP), ionomycin, and phorbol 12-myristate 13-acetate (PMA). In Ca^{2+}-containing media, the Ca^{2+} ionophore ionomycin in combination with PMA produced large increases in cytosolic Ca^{2+} activity and appeared to be the most effective stimulus for secretogranin II release [18]. This study also reported that astrocytes contain fewer smaller and less dense secretory granules containing secretogranin II, indicating that peptidergic granules in astrocytes are not uniform in morphological appearance.

One of the first peptides studied for exocytotic release from astrocytes was ANP. This peptide is a diuretic vasorelaxant hormone typically stored in specific secretory vesicles and is secreted from the heart atrial myocytes in response to overload and oxygen deficiency [7,43]. The function of ANP release from astrocytes, however, may play a role in cerebral blood regulation [54]. To study the discharge of ANP, Krzan et al. [47] transfected astrocytes with a construct to express pro-ANP fused with the emerald green fluorescent protein (ANP.emd). Transfection of astrocytes resulted in fluorescent puncta, representing vesicles. The number of puncta was reduced upon stimulating the cells by the Ca^{2+} ionophore ionomycin, which strictly depended on the extracellular Ca^{2+}. Concomitant with the Ca^{2+}-dependent decrease in fluorescent puncta, the fluorescence intensity of the FM 4–64 dye, a reporter of cumulative exocytosis, increased in a Ca^{2+}-dependent manner as well. Together these data strongly indicated that regulated exocytosis mediates the release of ANP from astrocytes. Interestingly, vesicles containing ANP also appear to contain ATP [61], which is consistent with the report that ATP is stored in secretorgranin II-containing vesicles [22].

In atrial myocytes, EM indicates that pro-ANP is condensed in the *trans*-Golgi network and, because pro-ANP is cleaved only on release, secretory vesicles budding off the *trans*-Golgi network are already mature. Their shape and size (120 to 175 nm) appears to be determined by the aggregation of the pro-ANP in vesicles [7]. In astrocytes the size of ANP recycling vesicles was studied by IEM after exposing live astrocytes to extracellular anti-ANP antibody, which sequestred within vesicles with diameters averaging 50 nm and ranging between 30 to 100 nm [72]. The mobility of these recycling ANP-containing vesicles was one order of magnitude smaller than that of ANP-containing vesicles trafficking to the plasma membrane vesicle docking site [70,71]. The clear-core nature and the smaller size of anti-ANP capturing vesicles in astrocytes relative to the values reported in atrial myocytes [7] is consistent with the observation that the vesicular ANP content determines the size and the shape of ANP-containing secretory vesicles [7].

The mobility of anti-ANP antibody capturing vesicles is dramatically reduced upon the stimulation of cells [72], which differs from the stimulation-increased mobility of anti-VGLUT1 antibody capturing vesicles in astrocytes [83]. The functional significance of these

observations is not clear, but the results clearly show that retrieving vesicle mobility is subject to the physiological state of the astrocyte [72]. This may play a role in the regulation of the vesicle cycle and vesicle cargo discharge [72]. It is possible to envision that arrested mobility of retrieving vesicles may affect the vesicle cargo discharge, at least by prolonging the interaction between the plasma membrane and the vesicle membrane. It was shown that the main mode of peptidergic vesicle exocytosis is the transient fusion (kiss-and-run), and that stimulation increases the frequency of occurrence of vesicle fusion as well as the dwell time of the established fusion pore and vesicle content discharge [84,89]. This mode of vesicle fusion would be facilitated, if the vesicle interaction with the cytoskeleton during retrieval is prevented or attenuated. The stimulation-induced vesicle mobility arrest is consistent with this view of vesicle cycle regulation; it increases the probability of peptide hormone discharge. In contrast, while such a mechanism may be related to peptidergic vesicles, in glutamatergic vesicles capturing the anti-VGLUT1 antibody, stimulation-induced enhanced post-fusion vesicle mobility may have a different function [83]. In this case, where the diffusional mobility of glutamate transmitter is orders of magnitude more mobile than the peptidergic hormones, the stimulation-increased vesicle mobility may terminate the glutamate discharge upon the reduction of interaction time between the vesicle and the plasma membrane. Furthermore, recycling vesicles may not only carry lumenal cargo but, also, membrane associated signalling molecules which participate in contact cell-to-cell interactions [45,81] or in determining the density of plasma membrane transporters [76], such as, the glutamate transporter EAAT2 [82].

Astrocytes also contain recycling vesicles, specialized endocytic compartments, which may serve bidirectional communication between neurons and glia. On one hand, these vesicles may take-up extracellular peptides, process them, and recycle them back into the extracellular space via secretory pathway and regulated exocytosis (Figure 3). When studying the activity-dependent secretion of BDNF and its extracellular availability, Bergami et al. [10] conducted very interesting experiments and provided evidence that BDNF, which is *de novo* synthetized in neurons, gets secreted after theta-burst stimulation in its pro-form into the extracellular medium. Then the pro-BDNF is rapidly internalized via the the pan-neurotrophin receptor $p75^{NTR}$ in perineuronal astrocytes via endocytosis, thereby restricting the availability of this neurotrophin at neuron-astrocyte contacts. After internalization, the pro-BDNF can undergo a recycling process, endowing astrocytes with the ability to resecrete this neurotrophin upon their stimulation. Ultrastructural IEM characterization revealed that BDNF fluorescently tagged with yellow fluorescent protein (BDNF-YFP) and gold particles labelled clear-core vesicles with 125 nm in diameter. Subsequently, the fluoresently labelled vesicles exposed their lumen to extracellular solution, presumably via a fusion pore, since the lumenal pH increased, detected as an increase in pH-dependent YFP fluorescence. To further investigate the entry of BDNF into the secretory pathway, astrocytes were preincubated with BDNF-YFP and their secretion was studied by stimulating cells with glutamate. The glutamate-evoked secretion of BDNF-YFP was inhibited, if cells were pretreated with tetanus neurotoxin, which cleaves Sb2. Co-localization between pro-BDNF and Sb2 confirmed that endocytosed pro-BDNF was routed into Sb2-containing vesicles. Taken together, this study shows that endocytic vesicles expressing $p75^{NTR}$ represent the main storage/recycling compartment for endocytosed pro-BDNF before routing it to the SNARE-dependent secretory pathway [10].

Concluding remarks

It is becoming clear that astrocytes are endowed with elements that qualify them as cells communicating with neurons and other cells in brain by employing regulated exocytosis. Astrocytes can synthesize and store gliotransmitters, i.e., amino acids, ATP and peptides, in SNARE-associated vesicles. The vesicular cargo discharge from these cells via regulated release occurs upon a delivery of physiological/pathological stimulus. It should be noted that

neighboring neurons and other cells rapidly sense and respond to the released gliotransmitters, albeit this subject was out of scope of the present review [reviewed in [59]]. Consequently, astrocytes appear as key players in central nervous system (patho)physiological processes. Further understanding of vesicular traffic to and from the plasma membrane via secretory pathway and within endocytic routes/recycling, as well as determining the location of exocytotic sites on astrocytes is of importance for astrocyte-neuron signaling. While astrocytic processes appear to be the ideal site for the location of vesicular fusions, exocytotic release can also occur on their bodies (reviewed in [56]). Although studies addressing these specific issues are only at the very beginning, there is palpable evidence that they will provide new insights into the understanding of how astrocytic membrane dynamics shape the signaling within the complex network of the brain tissue.

Abbreviations

ANP	atrial natriuretic peptide
ATP	adenosine 5'-triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BDNF	brain-derived neurotrophic factor
EGFP	enhanced green fluorescent protein
EM	electron microscopy
ER	endoplasmic reticulum
IP_3	permeant inositol 1,4,5-trisphosphate
IEM	immuno EM
NMDA	N-methyl-D-aspartate
PMA	phorbol 12-myristate 13-acetate
Sb2	synaptobrevin 2
SNAP-23	synaptosome-associated protein of 23 kDa
SNARE	the soluble N-ethyl maleimide-sensitive fusion protein attachment protein receptor
SOC	called store-operated Ca^{2+}
TCA	tricarboxylic acid
TIRF	total internal refection fluorescence
TRPC	canonical transient receptor potential
UTP	uridine 5'-triphosphate
VGLUT	vesicular glutamate transporter
V-ATPase	the vacuolar type of proton ATPase
YFP	yellow fluorescent protein
2-APB	2-aminoethyl ester

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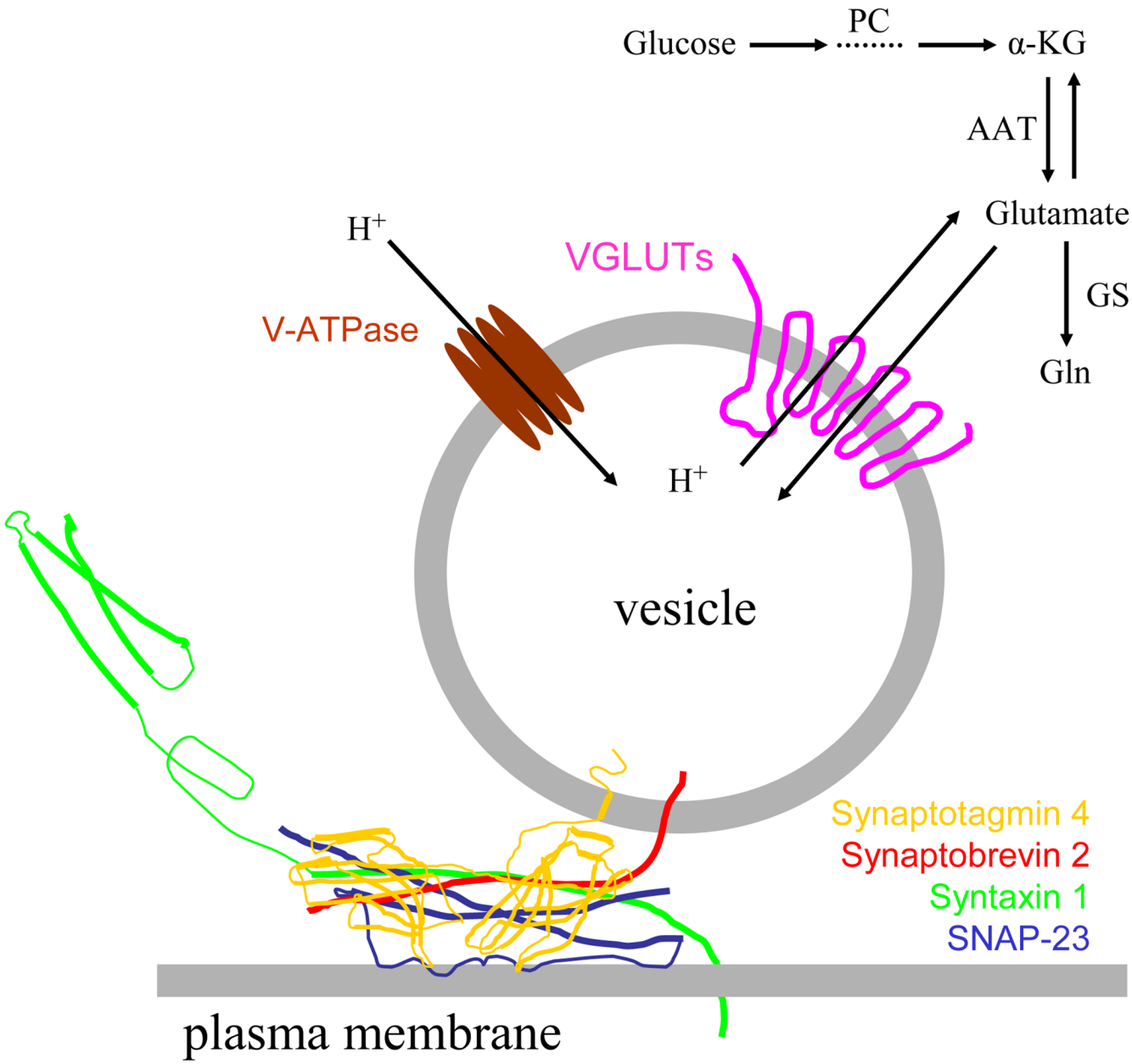

Figure 1.

Glutamate release by Ca^{2+}-dependent exocytosis. Glutamate packaged in vesicles is released from the astrocyte when the vesicle fuses with the plasma membrane. This fusion process is mediated by synaptotagmin 4 and SNARE proteins: syntaxin 1, synaptobrevin 2 and synaptosome-associated protein of 23 kDa (SNAP-23). Glutamate can be synthesized in astrocytes *de novo* from glucose entry to the tricarboxylic acid cycle via pyruvate carboxylase (PC). Glutamate is converted from the cycle intermediate, α-ketoglutarate (α-KG), usually by transamination of aspartate *via* aspartate amino transferase (AAT). The synthesized glutamate once in the cytosol can then be converted to glutamine (Gln) by glutamine synthetase (GS), or transported into vesicles via proton-dependent vesicular glutamate transporters (VGLUTs), especially isoform 3. The proton gradient is generated by vacuolar type H^+-ATPases (V-ATPase).

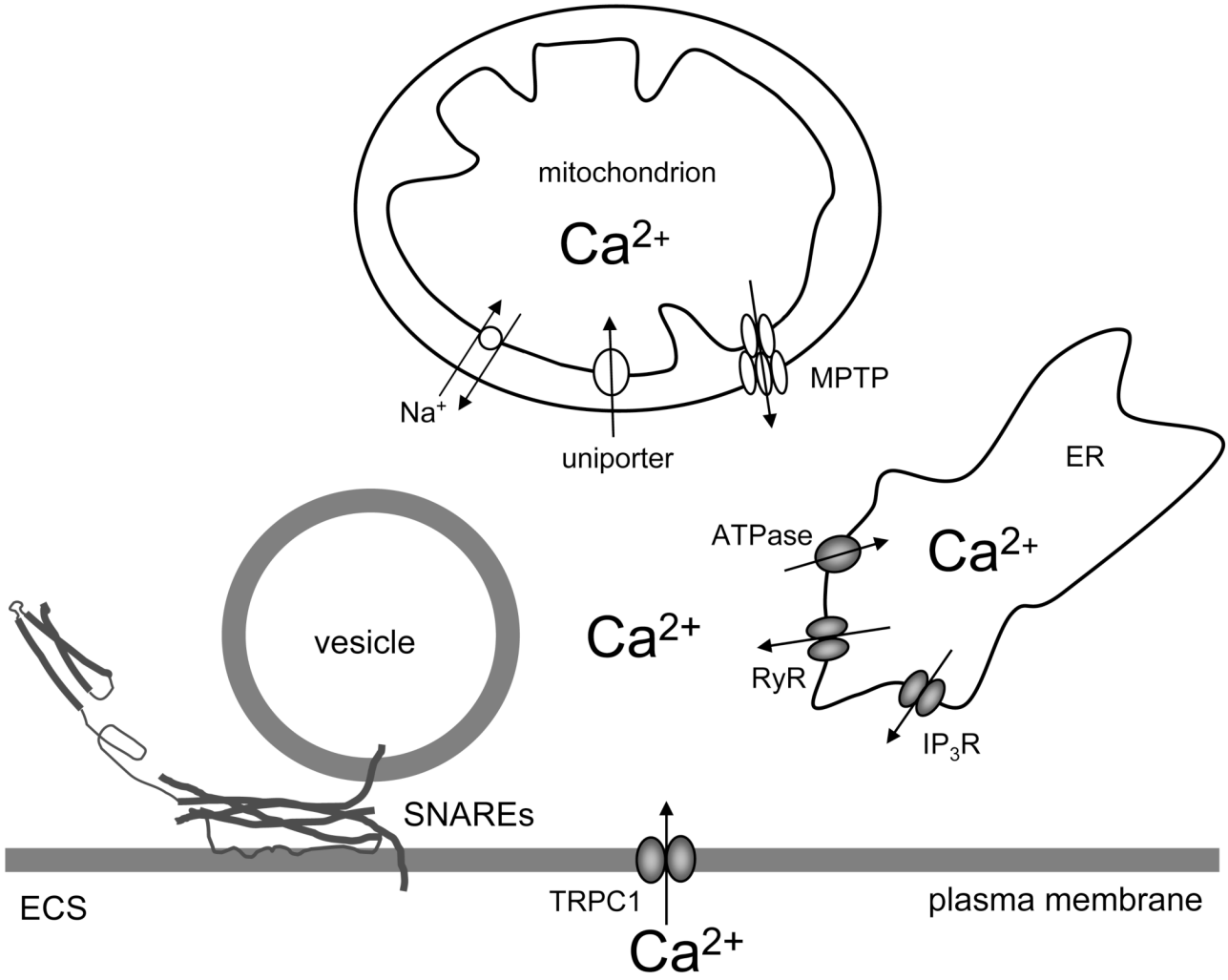

Figure 2.

Sources of cytosolic Ca^{2+} in vesicular release from astrocytes. Increase of cytosolic Ca^{2+} is sufficient and necessary to cause vesicular fusions and release of gliotransmitters. This process of regulated exocytosis requires the action of the ternary SNARE complex. Cytosolic Ca^{2+} accumulation could be caused by the entry of Ca^{2+} from the ER internal stores via IP_3 and ryanodine receptors (IP_3 and RyR). Store specific Ca^{2+}-ATPase fills these stores, although ultimately this action requires Ca^{2+} entry from the extracellular space (ECS) through canonical type 1 transient receptor potential (TRPC1). Mitochondrial Ca^{2+} uptake is mediated by the uniporter, while free Ca^{2+}within the mitochondrial matrix exits through the Na^+/ Ca^{2+} exchanger and the mitochondrial permeability transition pore (MPTP). Drawing is not to scale.

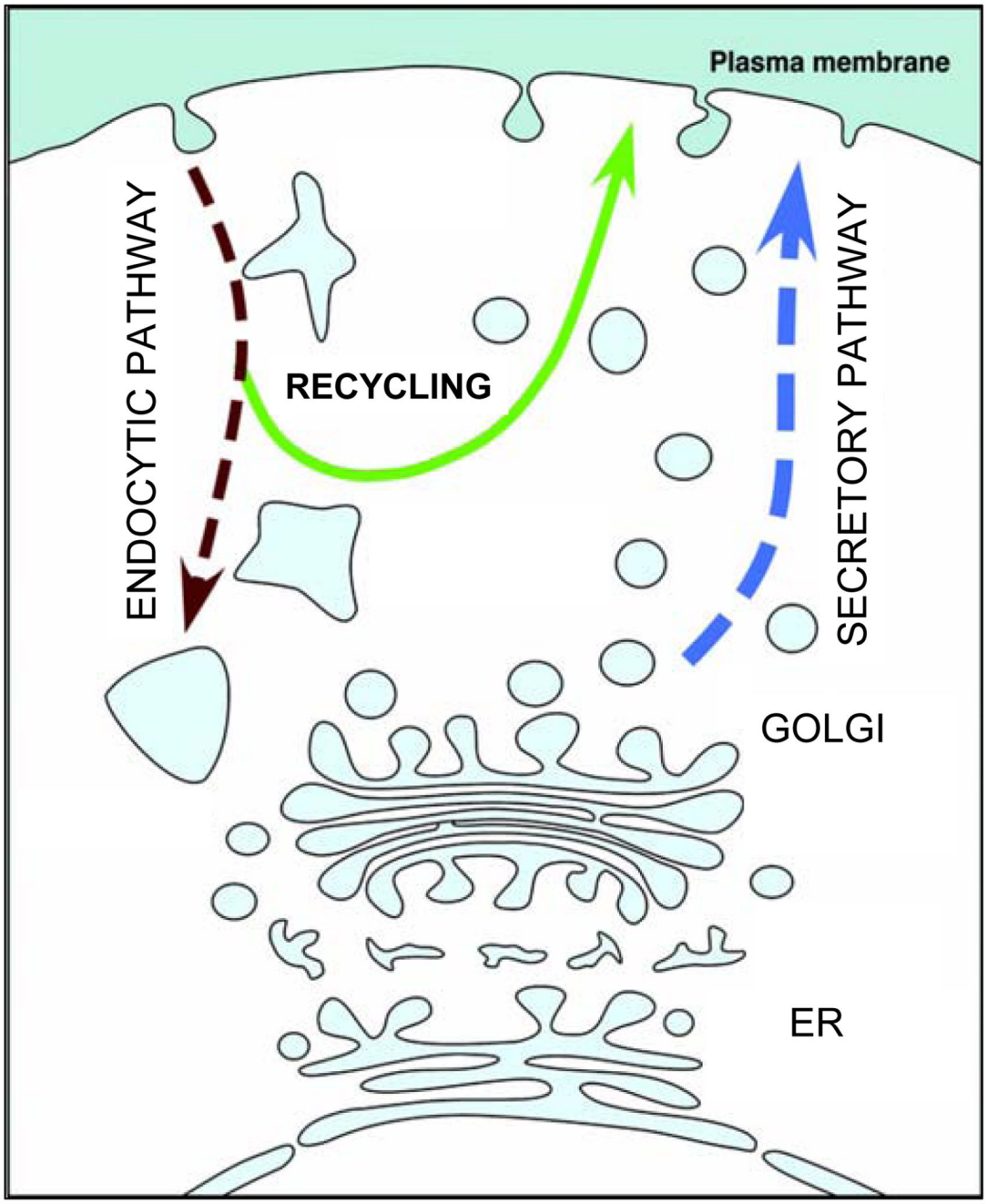

Figure 3.

Secretory pathway of peptidergic transmitters The pro-peptides are synthetized in the endoplasmic reticulum (ER). They then enter the Golgi compartments from which vesicles bud off, containing concentrated and sorted peptides. Secretory vesicles traffic away from the Golgi compartment along the secretory pathway to the plasma membrane where they dock and fuse with the plasma membrane upon a stimulus delivery, typically an increase in cytosolic Ca^{2+} levels/activity. Vesicles pinching off the plasma membrane via the endocytic pathway may be rerouted to the recycling pathway, where the substances captured from the extracellular space may be returned to the surface plasma membrane/extracellular space by entering regulated exocytosis of the secretory pathway.