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Regulated exocytosis in astrocytic signal integration

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Abstract

Astrocytes can be considered as signal integrators in central nervous system activity. These glial cells can respond to signals from the heterocellular milieu of the brain and subsequently release various molecules to signal to themselves and/or other neighboring neural cells. An important functional module that enables signal integration in astrocytes is exocytosis, a Ca^{2+} -dependent process consisting of vesicular fusion to the plasma membrane. Astrocytes utilize regulated exocytosis to release various signaling molecules stored in the vesicular lumen. Here we review the properties of exocytotic release of three classes of gliotransmitters: i) amino acids, ii) nucleotides and iii) peptides. Vesicles may not only carry luminal cargo, but also membrane associated molecules. Therefore, we also discuss exocytosis as a delivery mechanism for transporters and receptors to the plasma membrane, where these proteins are involved in astrocytic intercellular signaling.

Keywords

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

Introduction

The concept that astrocytes are multifunctional housekeeping cells subservient to neurons has largely been reconsidered. Besides maintaining the optimal environment for functioning of neurons (Nedergaard et al., 2003), evidence has emerged that astrocytes directly modulate processes such as synaptogenesis (Pfrieger and Barres, 1997), synaptic transmission (Araque et al., 1999; Newman, 2003), brain microcirculation (Anderson and Nedergaard, 2003; Zonta et al., 2003), short-term to long-term memory consolidation (Hertz and Gibbs, 2009), and immune responses (Fontana et al., 1984; Girvin et al., 2002). In this heterocellular signaling,

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astrocytes appear as signal integrators, since they generate outputs with variable timing in response to particular signals received from surrounding neural cells to affect neurons and/or other cellular components of the brain. A crucial element that facilitates the integrating functions of astrocytes is regulated exocytosis.

Astrocytes can release chemical messengers, gliotransmitters, into the extracellular space via Ca^{2+} -dependent exocytosis (Parpura et al., 1994), which has emerged as the prevalent release mechanism in these glial cells. Additionally, astrocytes express a variety of channels, receptors and transporters on their surface that mediate release of gliotransmitters: (i) anion channels (Pasantes Morales and Schousboe, 1988); (ii) unpaired connexons/pannexons, commonly referred to as “hemichannels” (Cotrina et al., 1998; Iglesias et al., 2009); (iii) ionotropic purinergic receptors (Duan et al., 2003); and (iv) transporters (Szatkowski et al., 1990; Warr et al., 1999; Rosenberg et al., 1994).

Exocytosis is an evolutionary trait of eukaryotic cells characterized by the formation of an aqueous channel, the fusion pore, upon the merger of vesicular and plasma membranes. The majority of ~200 cell types present in the human body perform exocytosis. It is through this process that various cells release vesicular chemical content into the extracellular space. Exocytosis represents one of the fastest biological events known. Increases in cytosolic Ca^{2+} can trigger exocytosis within a subfraction of a millisecond. However, such an exquisite speed can be reached only when vesicles are already pre-filled with transmitter, primed and docked to the plasma membrane, waiting for the cytosolic Ca^{2+} signal. In general, this is the case with vesicles filled with amino acids and/or nucleotides. Here, the delay is mainly determined by the spatio-temporal characteristics of the Ca^{2+} signaling mechanisms. However, in the case of vesicles filled with peptides, the delay between the trigger and the response may be significantly longer, in part because of relatively distant positioning of such vesicles to their plasma membrane fusion sites. Moreover, the repetitive/prolonged stimulation of peptidergic transmission requires peptide synthesis, packaging and anterograde vesicular trafficking.

Naturally, the delivery of secretory vesicles and fusion to the plasma membrane in astrocytes has been demonstrated in a number of studies. Crippa et al. (2006) expressed a chimeric protein, where enhanced green fluorescent protein (EGFP) was fused to the C-terminus of the vesicle membrane associated protein synaptobrevin 2 (Sb2-EGFP), in astrocytes. Since the C-terminus of Sb2 is located in the vesicular lumen, EGFP was expressed intravesicularly. When astrocytes were stimulated with a Ca^{2+} ionophore to increase cytosolic Ca^{2+} , many fluorescent Sb2-EGFP puncta vanished with a simultaneous enrichment 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 membrane capacitance (C_m). Certainly, an agonist-induced rise in astrocytic intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$) increased C_m , while concomitant measurements recorded a release of the gliotransmitter glutamate (Zhang et al., 2004b). Further evidence for vesicular exocytosis from astrocytes was provided by total internal reflection fluorescence (TIRF) microscopy (Bezzi et al., 2004; Domercq et al., 2006; Bowser and Khakh, 2007; Pangrsic et al., 2007; Marchaland et al., 2008; Pryazhnikov and Khiroug, 2008), where individual vesicular fusions were reported. As a consequence of vesicular fusions, quantal events of transmitter release, representing an exocytotic hallmark (Del Castillo and Katz, 1954), have been recorded from astrocytes (Pasti et al., 2001; Chen et al., 2005; Pangrsic et al., 2007). In this review, therefore, 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), neuropeptide Y (NPY), brain-derived neurotrophic factor (BDNF) and various cytokines/chemokines (Figure 1).

Secretory vesicles can also carry membrane-associated molecules. Delivery of membrane signaling proteins, receptors and transporters to the plasma membrane is of special interest for astrocytic interactions with other neural cells. Consequently, we also discuss intracellular vesicular traffic and/or exocytotic-mediated insertion of membrane proteins such as the excitatory amino acid transporter (EAAT) 2 and the G-protein coupled cannabinoid receptor 1 (CB1R) (Figure 1).

Exocytotic release of amino acids from astrocytes

Amino acid glutamate is synthesized within astrocytes as a by-product of the tricarboxylic acid (TCA) cycle. Glutamate is converted from the TCA cycle intermediate, α -ketoglutarate, usually via transamination of another amino acid, such as aspartate (Westergaard et al., 1996). Since astrocytes possess the enzyme pyruvate carboxylase, the synthesis of glutamate occurs *de novo* (Hertz et al., 1999). Through the action of serine racemase, an enzyme found predominately in astrocytes, D-serine is generated from L-serine, (Wolosker et al., 1999). These amino acid transmitters are stored intravesicularly. For their release into the extracellular space, they require increases in cytosolic Ca^{2+} concentrations, which can be caused by neuronal activity. Once released, they act upon neuronal receptors to modulate synaptic transmission (Ni et al., 2007; Oliet and Mothet, 2009).

Evidence for Ca^{2+} -dependent gliotransmitter release from astrocytes was initially demonstrated using high performance liquid chromatography to monitor glutamate release from cultured astrocytes (Parpura et al., 1994). Elevated $[\text{Ca}^{2+}]_i$ caused by the Ca^{2+} ionophore ionomycin was sufficient and necessary to cause glutamate release from astrocytes. Consistent with this finding, other stimuli that can increase astrocytic $[\text{Ca}^{2+}]_i$ including mechanical stimulation (Parpura et al., 1994; Araque et al., 1998b; Araque et al., 1998a; Innocenti et al., 2000; Hua et al., 2004; Montana et al., 2004), photostimulation (Parpura et al., 1994), photolysis of Ca^{2+} cages (Araque et al., 1998a; Parpura and Haydon, 2000; Zhang et al., 2004b), bradykinin (Parpura et al., 1994), prostaglandins (Bezzi et al., 1998) and ATP (Jeremic et al., 2001) all induce glutamate release. Furthermore, buffering of cytosolic Ca^{2+} capacity in astrocytes with the Ca^{2+} chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) reduces evoked glutamate release from astrocytes (Innocenti et al., 2000).

The majority of intracellular Ca^{2+} necessary for astrocytic glutamate release originates from endoplasmic reticulum (ER) stores (Figure 2). Depletion of internal Ca^{2+} stores with thapsigargin, a blocker of ER-specific Ca^{2+} -ATPase, blocks glutamate release from astrocytes (Araque et al., 1998b; Bezzi et al., 1998; Innocenti et al., 2000; Hua et al., 2004; Montana et al., 2004). Alkalinization of the cytosol, as occurs in the presence of ammonia, stimulates Ca^{2+} release from the ER, raising $[\text{Ca}^{2+}]_i$ levels, which induces glutamate release from astrocytes (Rose et al., 2005). Mechanical stimulation that causes increase of $[\text{Ca}^{2+}]_i$ levels results in glutamate release (Hua et al., 2004). This mechanically-induced glutamate release from astrocytes can be blocked by diphenylboric acid 2-aminoethyl ester (2-APB) solution, a cell-permeant inositol 1,4,5-trisphosphate (IP_3) receptor antagonist, implicating the role of IP_3 -sensitive internal stores in mediating Ca^{2+} -dependent glutamate release from astrocytes. Ryanodine/cafeine-sensitive ER stores play a role as well, since the treatment of astrocytes with ryanodine (at concentrations that block release of Ca^{2+} from ryanodine/cafeine-sensitive stores) attenuates mechanically-induced glutamate release. Furthermore, the sustained presence of caffeine, which depletes ryanodine/cafeine stores, also reduces mechanically-induced glutamate release. Thus, Ca^{2+} -dependent glutamate release from astrocytes involves both IP_3 - and ryanodine/cafeine-sensitive internal Ca^{2+} stores. (Hua et al., 2004). However, the functionality of ryanodine receptors in astrocytes is still debated since it has been reported that they lack activity in astrocytes *in situ* (Beck et al., 2004).

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 store-operated Ca^{2+} (SOC) channels, which become activated when ER Ca^{2+} is depleted (Takemura and Putney, 1989; Golovina, 2005). The precise mechanism governing $[\text{Ca}^{2+}]_i$ sensing by SOC channels remains elusive. However, astrocytes express canonical transient receptor potential (TRPC) channels implicated in SOC-mediated Ca^{2+} entry (Pizzo et al., 2001; Grimaldi et al., 2003; Golovina, 2005). Specifically, TRPC1 functionally contributes to Ca^{2+} -dependent glutamate release from astrocytes (Malarkey et al., 2008). Immunological blockade of the TRPC1 pore region in astrocytes significantly decreases SOC entry and mechanically-induced glutamate release. Besides SOC channels, voltage-gated Ca^{2+} channels can mediate entry of Ca^{2+} from the extracellular space. These channels regulate exocytotic glutamate release from astrocytes of the ventrobasal thalamus (Parri et al., 2001). Finally, ionotropic transmitter receptors can also mediate entry of Ca^{2+} into astrocytes [reviewed in (Verkhratsky, 2009)], but understanding the role of these receptors in exocytotic glutamate release from astrocytes requires further investigation.

Mitochondria, as a source/sink of intracellular Ca^{2+} , regulate exocytosis in astrocytes (Reyes and Parpura, 2008) (Figure 2). These organelles possess a Ca^{2+} uniporter that transports Ca^{2+} into the mitochondrial matrix when cytosolic $[\text{Ca}^{2+}]$ is greater than $\sim 0.5 \mu\text{M}$ (Miyata et al., 1991; Simpson and Russell, 1998). Blocking this uniporter with ruthenium 360, increases mechanically-induced cytosolic Ca^{2+} accumulation and glutamate release in cortical astrocytes. Conversely, blocking the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ exchanger with 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157) decreases mitochondrial Ca^{2+} efflux 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.

Ca^{2+} -dependent exocytosis depends on the presence of exocytotic secretory machinery (Figure 2). Indeed, astrocytes express proteins of the soluble N-ethyl maleimide-sensitive fusion protein attachment protein receptor (SNARE) complex: Sb2, also called vesicle-associated membrane protein 2 (VAMP2), VAMP3 (also referred to as cellubrevin), syntaxins 1, 2 and 4, synaptosome-associated protein of 23 kDa (SNAP-23), as well as several ancillary proteins to this complex, including synaptotagmin 4 and mammalian UNCoordinated 18 (Munc18) [(Paco et al., 2009) and reviewed in (Montana et al., 2006)]. The use of *Clostridial*, tetanus, and various types of botulinum toxins, which cleave SNARE proteins, reduces Ca^{2+} -dependent glutamate release in astrocytes [reviewed in (Montana et al., 2006)]. Additionally, the use of tetanus toxin, which cleaves astrocytic Sb2 and cellubrevin (Parpura et al., 1995), reduces exocytosis in astrocytes as reported by an attenuated increase in C_m (Kreft et al., 2004) and a reduction in the number of amperometric spikes (Chen et al., 2005). Accordingly, expression of the cytoplasmic domain of Sb2, which contains the SNARE domain but lacks the ability to anchor to the vesicular membrane, inhibits glutamate release from astrocytes (Zhang et al., 2004b). Similarly, expression of a mutated, dominant negative form of synaptotagmin 4 decreases Ca^{2+} -dependent glutamate release from astrocytes (Zhang et al., 2004a).

Proteins utilized for sequestering glutamate into vesicles have also been found in astrocytes. For example, astrocytes express vacuolar type H^+ ATPase (V-ATPase), which drives protons into the vesicular lumen, thereby generating the proton concentration gradient necessary for glutamate transport into vesicles (Wilhelm et al., 2004). V-ATPase inhibition with bafilomycin A_1 reduces glutamate release from astrocytes induced by various stimuli (Araque et al., 2000; Bezzi et al., 2001; Pasti et al., 2001; Montana et al., 2004; Crippa et al., 2006). Astrocytes also express the three known isoforms of VGLUTs: 1, 2 and 3, which use the proton gradient created by V-ATPases to package glutamate into vesicles (Freneau et al., 2002; Bezzi et al., 2004; Kreft et al., 2004; Montana et al., 2004; Zhang et al., 2004b; Anlauf and Derouiche,

2005; Crippa et al., 2006). Introduction of Rose Bengal, an allosteric inhibitor of VGLUTs, greatly reduces glutamate release, illustrating that these transporters are functional within astrocytes (Montana et al., 2004). VGLUT3 and the cytosolic concentration of glutamate are key limiting factors in Ca^{2+} -dependent release of glutamate from astrocytes (Ni and Parpura, 2009). Selective over-expression of individual VGLUT proteins in astrocytes showed that VGLUT3, but not VGLUT1 or VGLUT2, enhances mechanically-induced Ca^{2+} -dependent glutamate release. Similarly, inhibition of glutamine synthetase activity by L-methionine sulfoximine, which increases cytosolic glutamate concentrations, greatly potentiates mechanically-induced Ca^{2+} -dependent glutamate release from astrocytes without affecting intracellular Ca^{2+} dynamics (Ni and Parpura, 2009).

Secretory vesicles are the essential morphological elements needed for regulated Ca^{2+} -dependent exocytosis. Immunoelectron microscopy (IEM) experiments demonstrate that Sb2 is located in the vicinity of electron-lucent (clear) vesicular structures (Maienschein et al., 1999). Sb2-containing vesicles isolated from cultured astrocytes were heterogeneous in size, ranging from 30 to over 100 nm (Crippa et al., 2006). VGLUTs 1 and 2 in astrocytes *in situ* were associated with small, clear vesicles with a mean diameter of ~ 30 nm (Bezzi et al., 2004). Recycling glutamatergic vesicles that capture the extracellular antibody against VGLUT1 in a Ca^{2+} -dependent manner are electron-lucent and have a diameter of ~ 50 nm (Stenovec et al., 2007). Gliosomes are subcellular components of astrocyte processes isolated by fractionation and noted for their high rate of glutamate uptake (Nakamura et al., 1993). Vesicles found within gliosomes express Sb2 and VGLUT1. They are ~ 30 nm in diameter, and some are clathrin-coated (Stigliani et al., 2006). 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 (Kang et al., 2005; Xu et al., 2007). These vesicles can release glutamate, although they may represent a pharmacologically-induced phenomenon or may play a role in pathological processes (Bergersen and Gundersen, 2009). Overall, under physiologically relevant conditions Sb2 and VGLUTs are found in association with relatively small, clear vesicles in astrocytes.

Recycling of secretory vesicles at the plasma membrane has been demonstrated in astrocytes by several approaches. Application of ionomycin in the presence of extracellular Ca^{2+} causes uptake of the membrane recycling dye, FM 4-64 (Krzan et al., 2003). Similarly, stimulation of membrane recycling and consequent trapping of styryl dyes (FM 1-43 or FM 2-10) shows a punctate pattern of FM fluorescence in astrocytes, indicating vesicular uptake (Chen et al., 2005). As already mentioned, another approach that demonstrated vesicular uptake in astrocytes used extracellular antibodies against VGLUT1 that bind the luminal/intravesicular epitope of this transporter. After increasing cytoplasmic Ca^{2+} levels in the presence of anti-VGLUT1 antibodies, there was an increase in the number and intensity of intracellular fluorescent puncta in cultured astrocytes (Stenovec et al., 2007) as well as in astrocytes that reside within slices (Potokar et al., 2009).

Astrocytes can also release the amino acid D-serine (Schell et al., 1995), a co-agonist of the glycine binding site of the N-methyl-D-aspartate (NMDA) receptor. Mothet et al. (2005) showed that astrocytes release D-serine following glutamate receptor stimulation in a Ca^{2+} -dependent manner. This release was augmented by a Ca^{2+} ionophore and inhibited by application of thapsigargin. Furthermore, release of D-serine was reduced by concanamycin A, a V-ATPase inhibitor, and by 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 mechanism underlying Ca^{2+} -dependent release of D-serine from astrocytes was expanded in a subsequent study using confocal fluorescence microscopy (Martineau et al., 2008). Pharmacological inhibition of vesicular budding indicated that D-serine is packaged in astrocytes down stream of the Golgi

apparatus. In these experiments, Sb2-containing vesicles were recruited to the plasma membrane with a concomitant disappearance of intracellular D-serine punctate staining. The molecular identity of the vesicular transporter for D-serine remains undetermined. However, these studies suggest that D-serine is secreted from astrocytes via a regulated exocytosis/vesicular pathway.

The delay between a stimulus and fusion of vesicles containing amino acids was studied using electrophysiology and flash photolysis. As mentioned above, the rise in astrocytic $[Ca^{2+}]_i$, which results in an increase of C_m , leads to release of glutamate (Zhang et al., 2004b). Using flash photolysis of caged Ca^{2+} -compounds, Kreft et al. (Kreft et al., 2004) determined that at the $[Ca^{2+}]_i$ that triggers the half-maximal response rate of C_m increase (proportional to the rate of exocytosis) the delay was > 100 ms, more than two orders of magnitude longer than the delay in synaptic transmission (Sabatini and Regehr, 1999). Since active zones have not been detected in astrocytes, it may be that the observed relatively slow exocytotic responsiveness reflects the lack of distinct molecular organization of the exocytotic apparatus.

Exocytotic release of ATP from astrocytes

ATP is produced via glycolysis and oxidative phosphorylation. Once released into the extracellular space, ATP can mediate intercellular signaling by acting directly onto purinergic receptors. Alternatively, upon its hydrolysis by membrane-bound ecto-nucleotidases, the extracellular degradation products, ADP and adenosine, can activate distinct plasma membrane receptors [reviewed in (Fields and Burnstock, 2006)]. These pathways can be utilized by astrocytes to signal to adjacent neurons (Zhang et al., 2003; Pascual et al., 2005; Halassa et al., 2009) and microglia (Bianco et al., 2005; Davalos et al., 2005). As already outlined, astrocytes possess the necessary secretory machinery required for exocytosis. In addition to the smaller, clear core vesicles, astrocytes also contain large, dense core granules with diameters of ~ 115 nm, as evidenced by EM. These vesicles contain the secretory peptide secretogranin II (Calegari et al., 1999) and ATP (Coco et al., 2003). Based on immunoblotting, subcellular fractions containing secretogranin II were mainly distinct from fractions containing Sb2 (Calegari et al., 1999). However, dense core vesicles represent $\sim 2\%$ of the total number of immuno-isolated Sb2-containing vesicles (Crippa et al., 2006). It was demonstrated by IEM that Sb2 associates with some dense core vesicular structures with diameters ranging from 100-700 nm (Maienschein et al., 1999). Following subcellular fractionation and immunoblotting, Sb2, syntaxin 1, cellubrevin and synaptotagmin 1 were found to co-localize with ATP-containing organelles (Maienschein et al., 1999). It appears that there is some variability in the association of Sb2 with dense-core ATP-containing vesicles. It should be noted, however, that the presence of synaptotagmin 1 was not detected in astrocytes by others (Parpura et al., 1994; Zhang et al., 2004a; Crippa et al., 2006). The protein responsible for ATP accumulation in secretory vesicles has recently been identified as the vesicular nucleotide transporter (VNUT) SLC17A9 by immunocytochemistry in astrocytes (Sawada et al., 2008).

Morphological and biochemical evidence suggests that ATP may be released from astrocytes by Ca^{2+} -dependent exocytosis. In support of this idea, astrocytes exposed to nitric oxide demonstrated an increase in $[Ca^{2+}]_i$ and in the release of ATP into the extracellular space (Bal-Price et al., 2002). Buffering of intracellular Ca^{2+} with BAPTA or preventing vesicular release with botulinum toxin C greatly reduced ATP release. Furthermore, Coco et al. (2003) demonstrated that mechanically stimulated astrocytes release ATP, which is inhibited by application of bafilomycin A_1 or tetanus toxin. Interestingly, the reduction of ATP release by tetanus toxin was less pronounced than the reduction in glutamate release, indicating that ATP and glutamate release may be differentially regulated by distinct vesicular release machinery. Furthermore, expression of a dominant negative SNARE in astrocytes inhibits Ca^{2+} -induced exocytosis of ATP-containing vesicles (Pangrsic et al., 2007).

Under particular experimental conditions, the exocytotic release of ATP stored in astrocytic lysosomes has also been detected (Zhang et al., 2007). Incubation with FM recycling dyes stained astrocytic lysosomes based on co-localization of FM with various lysosomal markers under fluorescence microscopy. Regulated exocytosis of ATP-containing lysosomes in astrocytes was observed under TIRF microscopy, and was blocked by BAPTA. These lysosomes readily took up the fluorescent ATP analogue MANT-ATP which was also released upon stimulation. Indeed, the astrocytic lysosomal fraction, isolated by density gradient centrifugation, contained abundant ATP. Thus, it appears that ATP in astrocytes could be stored in and exocytotically released from at least two distinct organelles, dense-core granules and lysosomes. It will be necessary to determine the factors that dictate recruitment of these two distinct pools of organelles. For example, do the same organelles deliver ATP for release under physiological and pathological conditions or are there specific organelles that operate under particular conditions? An unexplored but exciting area of future research in respect to gliotransmission in general concerns specificity of SNARE isoforms in mediating vesicular versus lysosomal versus non-secretory exocytosis (Sorensen et al., 2003). In other words, is there functional redundancy in different SNARE protein isoforms or do they confer specificity in the type of contents released and/or in the particular exocytotic mechanism in astrocytes? Further studies are needed to address these questions.

To examine the kinetics of vesicular ATP release, Pryaznikov and Khiroug (2008) monitored the delay between the increase in $[Ca^{2+}]_i$ and vesicular release. To label ATP containing secretory vesicles, they incubated astrocytes with quinacrine. Using TIRF microscopy, quinacrine showed punctate staining. Increases in $[Ca^{2+}]_i$ reduced quinacrine fluorescent puncta, an indirect measurement of ATP release. Their results show that ATP release is delayed by several minutes and is highly asynchronous with the rise in $[Ca^{2+}]_i$; i.e. some vesicles exhibit relatively more rapid vesicle cargo discharge than others. These results obtained by optical monitoring of single vesicles are consistent with earlier results obtained in whole astrocytes by electrophysiological measurements (Kreft et al., 2004). The kinetics associated with lysosomal-mediated ATP release remain to be determined.

Exocytosis of peptides from astrocytes

In contrast to amino acids and ATP, which are loaded into vesicles by membrane transporters, peptidergic gliotransmitters enter vesicles via the synthetic secretory pathway. Pro-peptides are made in the ER, concentrated in Golgi compartments, and sorted into organelles such as lysosomes and vesicles. They are then processed to their final form before plasma membrane incorporation or release (Dannies, 1999) (Figure 1). Vesicles carrying peptidergic transmitters exhibit electron dense cores by EM and are termed dense-core vesicles, large dense-core vesicles or secretory granules. In general, their diameter is somewhat larger (~100 nm) in comparison with the small synaptic-like, clear-core vesicles (30-50 nm) and contain secretogranins (Winkler and Fischer-Colbrie, 1992). Secretogranin II is released upon stimulation by different secretagogues including bradykinin, cyclic adenosine monophosphate (cAMP), ionomycin, and phorbol 12-myristate 13-acetate (PMA) in a Ca^{2+} -dependent manner (Calegari et al., 1999). The specific SNARE isoforms involved in these processes in astrocytes have not been clearly defined.

ANP was one of the first peptides studied for exocytotic release from astrocytes, and may play a role in the regulation of cerebral blood flow (McKenzie et al., 2001). Transfection of astrocytes with a construct expressing pro-ANP fused with emerald green fluorescent protein (ANP.emd) results in accumulation of ANP.emd within secretogranin II-expressing secretory vesicles, represented as fluorescent puncta (Krzan et al., 2003; Paco et al., 2009). The number of puncta is reduced upon stimulation with ionomycin (Krzan et al., 2003). Concomitant with the Ca^{2+} -dependent decrease in fluorescent ANP.emd puncta, the fluorescence intensity of the

FM 4-64 dye, a reporter of cumulative exocytosis, also increases in a Ca^{2+} -dependent manner. Together, these data strongly indicate that Ca^{2+} -regulated exocytosis mediates the release of ANP from astrocytes. Furthermore, release of ANP from astrocytes is augmented in astrocytes that have been incubated in a cell-permeant form of cAMP, possibly due to increases in expression of vesicular release proteins such as synaptobrevin 2, syntaxin 1 and Munc18a (Paco et al., 2009). The vesicular content of secretogranin II is also increased in astrocytes incubated in cAMP. Interestingly, vesicles containing ANP also contain ATP (Pangrsic et al., 2007), which is consistent with the report that ATP is stored in secretogranin II-containing vesicles (Coco et al., 2003).

In atrial myocytes, pro-ANP-containing vesicles are ~ 120-175 nm in diameter and the vesicle size appears to be determined by the aggregation of pro-ANP in vesicles (Baertschi et al., 2001). In astrocytes, the diameter of ANP-recycling vesicles was assessed by IEM using extracellular ANP antibodies and ranged between 30 to 100 nm with an average of 50 nm. In addition to being smaller, these vesicles were clearer than ANP-containing vesicles released from atrial myocytes (Potokar et al., 2008). Because the vesicular ANP content determines the physical characteristics of ANP-containing vesicles, these differences may reflect differences in ANP content in releasing versus recycling vesicles. The mobility of recycling ANP-containing vesicles was one order of magnitude smaller than that of ANP-containing vesicles trafficking to the plasma membrane (Potokar et al., 2005; Potokar et al., 2007). Whether these differences in mobility are related to vesicular size, physiological state of the cell, or to intrinsic properties of releasing versus recycling vesicles remains unclear.

Interestingly, the mobility of ANP antibody-capturing vesicles is dramatically reduced by cell stimulation (Potokar et al., 2008), which differs from stimulation-increased mobility of VGLUT1 antibody-capturing vesicles in astrocytes (Stenovec et al., 2007). The full functional significance of these observations is not clear, but the results clearly show that retrieving-vesicle mobility is governed by vesicle-specific properties and by the physiological state of the astrocyte (Potokar et al., 2008). It is possible to envision that arrested mobility of peptidergic retrieving vesicles can enhance vesicle cargo discharge by prolonging the interaction between the plasma membrane and the vesicle membrane. It was shown that the main mode of peptidergic vesicle exocytosis is transient fusion (kiss-and-run), and that stimulation increases the frequency in vesicle fusion events, as well as the dwell time of the established fusion pore and vesicle content discharge (Stenovec et al., 2004; Vardjan et al., 2007). However, delayed retrieval may also make re-uptake more efficient, which can be important in the case of peptides, which require *de novo* synthesis, processing and packaging. In contrast, in glutamatergic vesicles capturing the anti-VGLUT1 antibody, stimulation-induced enhanced post-fusion vesicle mobility may have a different function (Stenovec et al., 2007). In this case, where the diffusional mobility of glutamate is orders of magnitude higher than peptidergic hormones, reduced interaction time between the vesicle and the plasma membrane would provide a regulatory control on the total amount of glutamate released. At the same time however, stimulation increases the number of fusion events and the overall effect of increased events but decreased interaction times has not been assessed.

Expression of neuropeptide Y (NPY), a peptide involved in synaptic transmission, was detected in astrocytes in brain slices from 8 day-old mice by immunocytochemistry and was confirmed by mass spectrometry of purified cortical astrocytes (Ramamoorthy and Whim, 2008). NPY immunoreactivity colocalized with GM130, a marker of the *cis*-Golgi, and with carboxypeptidase E, an enzyme that processes prohormones, indicating that NPY is expressed in appropriate regions of the regulated secretory pathway. NPY-expressing vesicles were larger and physically distinct from VGLUT1-expressing vesicles. Glutamate induced NPY release from astrocytes, represented as a step-like decrease in fluorescence intensity of NPY-red fluorescent protein puncta. This effect was mediated via activation of group I metabotropic glutamate

receptors and depended on release of Ca^{2+} from intracellular stores. Glutamate, but not ATP, bradykinin or histamine, induced NPY release from astrocytes. This finding is intriguing because all four of these secretagogues cause increases in $[\text{Ca}^{2+}]_i$, suggesting that regulation of peptide release requires additional mechanisms that are as yet undetermined.

The specialized endocytic compartments that mediate vesicle recycling in astrocytes may serve to facilitate bidirectional communication between neurons and glia. These vesicles can take up extracellular peptides and recycle them back into the extracellular space via the secretory pathway and regulated exocytosis. When studying activity-dependent secretion of BDNF and its extracellular availability, Bergami et al. (2008) demonstrated that BDNF, which is *de novo* synthesized in neurons, is secreted extracellularly after theta-burst stimulation in its pro-form into the extracellular medium. Pro-BDNF is then rapidly endocytosed via the pan-neurotrophin receptor p75^{NTR} in perineuronal astrocytes, thereby restricting the availability of this neurotrophin at neuron-astrocyte contacts. BDNF- yellow fluorescent protein (BDNF-YFP) labeled clear-core vesicles that were ~ 125 nm in diameter. Furthermore, they showed by IEM that internalized pro-BDNF is recycled and secreted from astrocytes. This secretion was enhanced by application of extracellular glutamate. The glutamate-evoked secretion of BDNF-YFP was inhibited if cells were pretreated with tetanus toxin. 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 (Bergami et al., 2008).

Astrocytes can also produce chemokines that directly modulate synaptic activity. Under neuroinflammatory conditions, activated astrocytes, as well as other immune cells in the CNS, can produce cytokines and chemokines such as interferon- α/β , ; interleukin(IL)-6, IL-8, IL-10, IL-12/IL-23, IL-17, IL-27, chemokine (C-C motif) ligand (CCL)2, CCL3, CCL4, CCL5, CCL10, chemokine CXC motif ligand (CXCL)10, CXCL12, insulin-like growth factor-1, transforming growth factor- β , tumor necrosis factor- α (TNF- α), granulocyte macrophage-colony stimulating factor and prostaglandin E_2 (PGE_2)(Montgomery, 1994; Constantinescu et al., 2005; Koizumi et al., 2005; Baker et al., 2009). Very few studies have addressed the potential role of cytokine and chemokine expression in modulating exocytosis and synaptic activity. However, one intriguing study demonstrated that activation of CXC receptor 4 (CXCR4), either by CXCL12 or by the human immunodeficiency virus peptide gp120, induces release of TNF- α from astrocytes and microglia (Bezzi et al., 2001). TNF- α , acting through TNF receptor 1, induced glutamate release from astrocytes, but not from microglia. In these studies, TNF- α induced production of PGE_2 , which increased $[\text{Ca}^{2+}]_i$ and induced glutamate release from astrocytes. Excessive glutamate release can induce neuron cell death via excitotoxicity. Glutamate released from astrocytes can directly induce TNF- α production from microglia, providing a feed-forward mechanism of inflammation-induced excitotoxicity. Indeed, it was demonstrated that gp120-induced TNF- α production and subsequent glutamate release induced excitotoxic neuron death (Bezzi et al., 2001). TIRF studies demonstrated that CXCR4-mediated glutamate release occurs on the order of a few hundred milliseconds, which is longer than for synaptic vesicle release but faster than dense-core granule release from neurosecretory cells (Cali et al., 2008). CXCR4 is expressed on presynaptic terminals of neurons as well as on astrocytes (Banisadr et al., 2002; Tran et al., 2007). Additionally, when released from astrocytes, TNF- α can cause an increase in the surface expression of neuronal α -amino-3-hydroxy-5-methyl-isoxazole propionate (AMPA) receptors, and, thus, this factor plays a role in controlling synaptic strength (Beattie et al., 2002). Therefore, these results demonstrate how astrocytes may serve as integrators that recognize and respond to environmental signals, such as chemokines, and generate neuromodulatory responses, such as glutamate release and regulation of surface receptor expression.

Exocytosis as the mechanism for delivery of the plasma membrane signaling proteins

Vesicles may not only carry luminal cargo, but also plasma membrane transporters (Figure 1) such as glutamate transporters (Robinson, 2002) which take up glutamate from the extracellular space [reviewed in (Danbolt, 2001)]. Mennerick and Zorumski (Mennerick and Zorumski, 1994) demonstrated that neurons can signal to astrocytes through the synaptic release of glutamate, which activates astrocytic glutamate uptake systems. In turn, astrocytic uptake of glutamate from the extracellular space modulates the kinetics of synaptic neurotransmission. It should be noted that pathophysiological events, such as ischemia, may favor transporters to operate in reverse, so that astrocytes can also utilize glutamate transporters as a mechanism of release (Szatkowski et al., 1990). Thus, there is a need for studying the traffic of EAAT in astrocytes. One study demonstrated that transfection of EAAT2 tagged with EGFP into astrocytes predominantly labels the plasma membrane (Stenovec et al., 2008). Ionomycin induces exocytosis in these cells, which correlates with local increases or decreases in plasma membrane fluorescence, indicating Ca^{2+} -induced trafficking of EAAT2-EGFP. Furthermore, stimulation of astrocytes with PMA induces internalization of EAAT2-EGFP. This internalization is blocked by co-transfection of EAAT2-EGFP with a dominant negative form of dynamin, suggesting that internalization is likely clathrin-mediated. One can envision that similar mechanisms exist to regulate trafficking of transmembrane receptors as well.

Astrocytes possess a multitude of G-protein-coupled receptors (GPCR) [reviewed in e.g., (Verkhatsky, 2009)] that respond to signaling molecules released by neurons and themselves, including the CB1R (Rodriguez et al., 2001), which is thought to mediate neuron-astrocyte communication. Navarrete and Araque (Navarrete and Araque, 2008) demonstrated that in hippocampal slices depolarization of neurons leads to release of endocannabinoids that in turn signal to adjacent astrocytes by activating CB1Rs. This causes an increase in the astrocytic $[\text{Ca}^{2+}]_i$, triggering stimulated glutamate release, which then activates NMDA receptors on nearby neurons. The existence of this endocannabinoid-glutamate signaling pathway underscores the importance of understanding the CB1R vesicular traffic in astrocytes. Protein trafficking plays an important role in regulating CB1R expression with receptors changing localization between the plasma membrane and intracellular compartments. In certain cell types at rest, CB1R is constitutively endocytosed leading to a predominantly intracellular localization (Leterrier et al., 2004; McDonald et al., 2007). Therefore, endocytic trafficking can be an important regulator of CB1R availability at the plasma membrane. The characteristics of constitutive CB1R trafficking within astrocytes have been recently investigated (Osborne et al., 2009) using recombinant fluorescent protein chimeras of the CB1R. Whether intracellular Ca^{2+} dynamics affect trafficking of CB1R and its delivery to the plasma membrane remains to be determined. However, vesicular (intracellular) CB1 receptors can still engage cell signaling (Gomes et al., 2009) which may be activated by lipophilic (membrane-permeant) cannabinoid ligands or via innate, constitutive activity. Clearly, further studies are necessary to elucidate the mechanisms governing the trafficking of plasma membrane transporters and receptors.

Concluding remarks

Astrocytes can receive signals from neighboring neural cells and respond to these inputs with various delays. An important functional module that can contribute to the variable responsiveness is exocytosis, representing the fusion of vesicle membrane with the plasma membrane (Table 1 summarizes properties of vesicular release from astrocytes). Astrocytes can synthesize and store gliotransmitters, i.e., amino acids, ATP and peptides, in SNARE-associated vesicles. Moreover, exocytosis participates in the release of gliotransmitters as well as in the insertion of membrane signaling proteins into the plasma membrane.

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Abbreviations

ANP	atrial natriuretic peptide
ATP	adenosine 5'-triphosphate
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BBB	brain barrier
BDNF	brain-derived neurotrophic factor
[Ca ²⁺] _i	intracellular calcium ion concentration
cAMP	cyclic adenosine monophosphate
CB1R	cannabinoid receptor 1
CCL	chemokine (C-C motif) ligand
C _m	membrane capacitance
CNS	central nervous system
CXC	chemokine CXC motif
CXCL	CXC ligand
CXCR	CXC receptor
EGFP	enhanced green fluorescent protein
EAAT	excitatory amino acid transporter
EM	electron microscopy

ER	endoplasmic reticulum
IP ₃	inositol 1,4,5-trisphosphate
IEM	immuno EM
IL	interleukin
Munc	mammalian UNCoordinated
NMDA	N-methyl-D-aspartate
NPY	neuropeptide Y
PGE ₂	prostaglandin E ₂
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	store-operated Ca ²⁺
TCA	tricarboxylic acid
TIRF	total internal reflection fluorescence
TRPC	canonical transient receptor potential
VGLUT	vesicular glutamate transporter
V-ATPase	the vacuolar type of proton ATPase
YFP	yellow fluorescent protein
2-APB	2-aminoethyl ester

Figure 1.

Regulated exocytosis in astrocytes. Ca^{2+} -dependent exocytosis is a mechanism underlying the release of three classes of gliotransmitters: amino acids (AA), nucleotides (e.g., ATP), and peptides from astrocytes. These chemical transmitters are stored in and released from at least two distinct classes of vesicles (clear and dense core) at the plasma membrane. Secretory vesicles can also carry membrane-associated molecules. Delivery of membrane signaling receptors, such as the G-protein coupled receptors (GPCRs) and transporters, such as the excitatory amino acid transporters (EAATs) to the plasma membrane is of special interest for astrocytic interactions with other neural cells. Arrows indicate direction of vesicle trafficking. Dashed arrow indicates the presumed recycling of ATP/peptide, GPCR, and EAAT containing vesicles. Ext, extracellular space; Int, intracellular space; Nuc, nucleus. Drawing is not to scale.

Figure 2.

Multiple sources of cytosolic Ca^{2+} that contribute to vesicular release from astrocytes. Vesicles (ves) fuse to the plasma membrane and release gliotransmitters. This process of regulated exocytosis is governed by the action of the ternary SNARE complex and is triggered by a preceding increase of cytosolic Ca^{2+} . Cytosolic Ca^{2+} accumulation is predominately caused by the entry of Ca^{2+} from endoplasmic reticulum (ER) internal stores via ryanodine and inositol 1,4,5-trisphosphate receptors (RyR and IP₃R, respectively). Store-specific Ca^{2+} -ATPase (SERCA) fills these stores, which requires Ca^{2+} entry from the extracellular space (Ext) through store-operated Ca^{2+} channels (SOC) located at the plasma membrane. Mitochondria (Mito) represent a source/sink of cytosolic Ca^{2+} ; uptake is mediated by the uniporter, efflux occurs via the Na⁺/Ca²⁺ exchanger and the mitochondrial permeability transition pore (MPTP). Int, intracellular space; Nuc, nucleus. Drawing is not to scale.

Table 1

Properties of vesicular release from astrocytes.

Vesicle Cargo	Release Latency	Vesicle Appearance	Vesicle Size	Recycling
Amino Acids	milliseconds	clear core	~ 30 - 50 nm	fast
Nucleotides	seconds	dense, not so dense, and clear core	~ 100 nm	presumably fast
Peptides	seconds	dense and not so dense core	~ 30-100 nm	slow

Note: Vesicular size in astrocytes seems to substantially vary (see text for details)