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LOOSE EXCITATION-SECRETION COUPLING IN ASTROCYTES

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Abstract

Astrocytes play an important housekeeping role in the central nervous system. Additionally, as secretory cells, they actively participate in cell-to-cell communication, which can be mediated by membrane-bound vesicles. The gliosignaling molecules stored in these vesicles are discharged into the extracellular space after the vesicle membrane fuses with the plasma membrane. This process is termed exocytosis, regulated by SNARE proteins, and triggered by elevations in cytosolic calcium levels, which are necessary and sufficient for exocytosis in astrocytes. For astrocytic exocytosis, calcium is sourced from the intracellular endoplasmic reticulum (ER) store, although its entry from the extracellular space contributes to cytosolic calcium dynamics in astrocytes. Here, we discuss calcium management in astrocytic exocytosis and the properties of the membrane-bound vesicles that store gliosignaling molecules, including the vesicle fusion machinery and kinetics of vesicle content discharge. In astrocytes, the delay between the increase in cytosolic calcium activity and the discharge of secretions from the vesicular lumen is orders of magnitude longer than that in neurons. This relatively loose excitation-secretion coupling is likely tailored to the participation of astrocytes in modulating neural network processing.

Keywords

calcium homeostasis; GPCR; vesicular release; exocytosis; astrocyte secretion; SNAREs

Introduction

Astrocytes have been considered secretory cells for over a century. In 1910, Jean Nageotte proposed, based on his microscopic observations, that glial cells (astroglia in particular) act as secretory elements in the central nervous system (CNS) (Nageotte 1910). This hypothesis has been experimentally confirmed in the last two decades, and it is now well accepted that information processing is not a task exclusively dedicated to neurons but shared by

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astrocytes (Rusakov et al. 2011; Verkhratsky et al. 1998), which are abundant and arguably the most heterogeneous glial cells in the CNS.

A single astrocyte may be associated with a large number of neurons and their synaptic contacts. Approximately 213 synapses/100 μm^3 exist in the adult rat hippocampal CA1 region (Kirov et al. 1999). Because the estimated volume of a rat astrocyte is 66,000 μm^3 , a single astrocyte in the rat hippocampus can be associated with up to 140,000 synapses (Bushong et al. 2002). Human hippocampal astrocytes are larger and more complex, with a volume approximately 27 times greater than that of their rodent counterparts. Consequently, a single human astrocyte may be associated with up to two million synapses (Oberheim et al. 2006), suggesting an evolutionary expansion of the magnitude of astrocyte–neuron interactions (Han et al. 2013). Abundant astroglial interactions with neurons are not restricted to the hippocampus. For instance, a single cortical astrocyte enwraps 4–8 neuronal bodies and 300–600 dendrites (Halassa et al. 2007). Due to their close association with synapses, astrocytes not only respond to but also influence neuronal signaling. Hence, these glial cells receive humoral signals from the synaptic cleft and release their own signaling molecules called gliotransmitters (Haydon 2001; Vesce et al. 1999; Zorec et al. 2012), which are chiefly the same chemicals released by neurons (e.g., glutamate). Regardless of some skepticism (Fujita et al. 2014; Sloan and Barres 2014), bidirectional astrocyte–neuron signaling is well accepted as a type of heterocellular signaling that occurs at a morpho-functional unit termed the tripartite synapse (Araque et al. 1999; Perea et al. 2009). Within this structure, astrocytes are thought to signal relatively slow (tens of milliseconds to seconds) compared to neurons (submilli- to milliseconds). The rate of this signaling is, at least in part, limited by the availability of metabolic precursors for gliotransmitters and structural remodeling (Vardjan et al. 2014b). However, some CNS functions linked to glia, including neurodevelopment, memory maintenance, neuroprotection, and homeostatic metabolic mechanisms involve much slower signaling processes (minutes to hours) compared to astrocyte–neuron signaling at the level of a tripartite synapse (Araque et al. 1998). The comprehensive glial communication system encompassing such wide time scales could be designated to the gliocrine system (Vardjan and Zorec 2015), a term coined by analogy to the endocrine system, as the endocrine system provides homeostatic control of bodily functions primarily on a slower time scale than the very rapid neuronal synaptic signaling within the nervous system (Vardjan and Zorec 2015). Moreover, similar to the endocrine system in which hormones and chemical signals released into blood are transported by convection/circulation to their targets, gliosignaling molecules released by the gliocrine system into the extracellular milieu of brain parenchyma can be transported by the convective glymphatic system (Thrane et al. 2014). The above mentioned differences in spatial and temporal dimensions between astrocytes and neurons may enable astrocytes to act as integrators in the CNS, as proposed when the first membrane capacitance measurements in astrocytes were performed ((Kreft et al. 2004), reviewed in (Vardjan and Zorec 2015)), and to fine tune the synaptic circuitry in respect to the current environmental state (reviewed in (Araque et al. 2014)).

Unlike the well-characterized vesicular exocytosis of neurons and neuroendocrine cells, the mechanisms of gliosignaling molecule release from astrocytes are still elusive, and are perhaps more diverse (Bezzi et al. 2004; Hamilton and Attwell 2010; Li et al. 2013; Li et al.

2008; Malarkey and Parpura 2008). Nonetheless, astrocytes were shown, using a variety of experimental approaches that included optical methods, membrane capacitance measurements, electrochemical amperometry, and selective interference with proteins of the exocytotic machinery (Guek et al. 2012; Parpura et al. 1994), to release chemical messengers *in vitro* and *in situ* through a Ca^{2+} -dependent vesicle-based mechanism (i.e., exocytosis) under physiological conditions. In addition to vesicular/exocytotic mechanism(s), non-vesicle-based mechanisms of release from astrocytes were reported to occur (1) through plasmalemmal channels, including swelling-induced opening of volume-regulated anion channels, connexons/pannexons (hemichannels), ionotropic pore-forming P2X_7 purinergic receptors, the two-pore-domain potassium channel Trek-1, or Bestrophin-1 channels (Woo et al. 2012), and (2) via plasmalemmal transporters, such as the reversal uptake by plasma membrane excitatory amino acid (glutamate) transporters, the (hetero)exchange via the cystine–glutamate antiporter or organic anion transporters (reviewed in (Malarkey and Parpura 2008). These non-vesicular release mechanisms, with the exception of Bestrophin-1, are Ca^{2+} -independent and are likely activated under pathological conditions (Agulhon et al. 2008; Hamilton and Attwell 2010).

In this review, we present the current knowledge on the astrocytic vesicular release of gliosignaling molecules, which encompass amino acids (glutamate; (Bezzi et al. 2004; Parpura et al. 1994; Zhang et al. 2004b), D-serine (Martineau et al. 2008; Mothet et al. 2005), peptides (atrial natriuretic peptide [ANP]; (Krzan et al. 2003), secretogranin II, chromogranin (Calegari et al. 1999), and nucleotides (adenosine 5'-triphosphate [ATP]; (Bal-Price et al. 2002; Coco et al. 2003; Pangrsic et al. 2007; Zhang et al. 2007). First, we discuss the molecular machinery underlying the exocytosis-based mechanisms along with the characteristics of different vesicle types that store transmitter/gliosignaling molecules in astrocytes. A description follows of astrocytic cytosolic excitability, based on intracellular calcium activity variations, that governs vesicular fusion events and secretion. Finally, we present the kinetics of astrocytic exocytotic release, closing with an overarching conclusion that the excitation-secretion coupling in astrocytes is loose, that is, a relatively long delay occurs between the stimulus and secretion compared with that in endocrine and neuronal cells.

Astrocytes express proteins necessary for SNARE-dependent vesicular fusion

Neuronal signaling involves the propagation of an action potential down the axon to the synaptic terminal, where synaptic vesicles are docked and primed at the vesicle release zones/active zones of the presynaptic terminals. Upon calcium elevation in the terminal, the vesicles fuse, an event mediated by the calcium sensor synaptotagmin 1 located at vesicles and by the soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins, that is, synaptosomal-associated protein of 25 kDa (SNAP25), syntaxin, and vesicle-associated membrane protein 2 (VAMP2), also referred to as synaptobrevin 2; SNAP25 and syntaxin are enriched at the plasma membrane, whereas VAMP2 is chiefly present at the vesicular membrane (Südhof 2013). Electron microscopy studies showed that astrocytes lack the structurally organized vesicle release zones that can be observed in

presynaptic neurons (Bezzi et al. 2004; Jourdain et al. 2007). However, similar to neurons, astrocytes express SNARE and its associated proteins, such as synaptotagmins and Sec1/Munc18-like proteins (reviewed in (Montana et al. 2006), although the SNARE components of the exocytotic apparatus between astrocytes and neurons are not identical (e.g., astrocytes utilize SNAP23, whereas neurons use SNAP25; (Montana et al. 2006), and the number of these molecules in a single vesicle differs (e.g., approximately 70 copies of VAMP2 are in neuronal vesicles as opposed to 25 in astrocytes; (Singh et al. 2014)).

The first evidence for the presence of a SNARE exocytotic apparatus in astrocytes was shown by immunocytochemistry and Western blot studies using cultured astrocytes (Parpura et al. 1995). Further data obtained on cultured and freshly isolated astrocytes showed that astrocytes express a variety of SNARE proteins, including the R-SNARE (vesicular SNARE) proteins synaptobrevin 2/VAMP2, cellubrevin/VAMP3 (Araque et al. 2000; Crippa et al. 2006; Jeftinija et al. 1997; Maienschein et al. 1999; Martineau et al. 2008; Mothet et al. 2005; Parpura et al. 1995; Wilhelm et al. 2004), and tetanus neurotoxin (TeNT)-insensitive VAMP (TI-VAMP)/VAMP7 (Verderio et al. 2012), as well as the Q-SNARE (target membrane SNARE) proteins SNAP23 and syntaxins 1, 2, 3, and 4 (Hepp et al. 1999; Paco et al. 2009; Zhang et al. 2004b). In addition to the SNARE proteins, cultured astrocytes also express SNARE-associated proteins, such as isoforms of Munc18 (Paco et al. 2009) and synaptotagmin 4 (Zhang et al. 2004a). In mammalian systems, unlike in *Drosophila*, synaptotagmin 4 is not the Ca^{2+} -sensor for regulated exocytosis (Wang and Chapman 2010) but appears to be important for modulating Ca^{2+} -evoked exocytosis (Südhof 2012). The cleavage of SNARE proteins with clostridial toxins, TeNT and various types of botulinum neurotoxins (BoNT), reduces glutamate release in cultured astrocytes as well as exocytosis measured as attenuated membrane capacitance (C_m) increases (Kreft et al. 2004) and decreases in amperometric spikes, further indicating that astrocytes in culture possess proteins obligatory for regulated exocytosis. Moreover, the SNARE proteins VAMP2 and VAMP3 colocalize with vesicles storing ATP (Maienschein et al. 1999) or D-serine (Martineau et al. 2008) in cultured astrocytes.

Cultured astrocytes differ in many respects from astrocytes *in situ* (in surrounding environment, morphology, protein expression, etc.) and *in vivo*, and may also differ in the expression of SNARE proteins. *In situ* studies using immunogold cytochemistry and confocal microscopy confirmed the presence of the SNARE proteins VAMP2 (Wilhelm et al. 2004), VAMP3 (Bergersen and Gundersen 2009; Bezzi et al. 2004; Jourdain et al. 2007; Schubert et al. 2011; Zhang et al. 2004b), TI-VAMP/VAMP7 (Verderio et al. 2012), SNAP23 (Schubert et al. 2011), and syntaxin 1 (Schubert et al. 2011) in tissue astrocytes. VAMP3 was shown to colocalize with the vesicular glutamate transporters VGLUTs 1 and 2 on small synaptic-like microvesicles (SLMVs) storing glutamate (Bergersen and Gundersen 2009; Bezzi et al. 2004; Jourdain et al. 2007; Zhang et al. 2004b) and likely D-serine (Martineau et al. 2013). Moreover, TI-VAMP/VAMP7 colocalizes with the markers of late endocytic/lysosomal compartments (Verderio et al. 2012) that store ATP (Bal-Price et al. 2002; Coco et al. 2003; Pangrsic et al. 2007; Zhang et al. 2007), an important gliosignaling molecule. Inactivation of VAMP2 or VAMP3 in astrocytes by TeNT abolishes the release of glutamate or D-serine from astrocytes *in situ* (Henneberger et al. 2010; Jourdain et al. 2007;

Perea and Araque 2007), implying SNARE-mediated release of these transmitters. Additionally, a mouse model that allows expression of a dominant negative SNARE transgene in astrocytes was generated to interfere specifically with VAMP2 and 3 in astrocytes (Halassa et al. 2009; Pascual et al. 2005). Use of these mice revealed that synaptic transmission and plasticity were altered (Hines and Haydon 2013; Lalo et al. 2014; Nadjar et al. 2013; Pascual et al. 2005; Turner et al. 2013), suggesting the involvement of astrocytic VAMP2/3-dependent exocytosis in information transfer in the brain. Moreover, disruption of the calcium-dependent vesicular glutamate release from Müller cells, a subtype of astroglia that express BoNT/B-sensitive VAMP2/3, was observed in bigenic mice with targeted expression of BoNT/B in Müller cells (Slezak et al. 2012). *In vivo* recordings in mice with inducible expression of TeNT in astrocytes revealed that TeNT-sensitive vesicular release from astrocytes is required for sustaining gamma oscillations associated with recognition memory in mice (Lee et al. 2014). Studies examining mRNA in brain tissue detected co-expression of several synaptotagmin isoforms, including synaptotagmins 1, 4, 7, and 11. The latter was most abundantly expressed in astrocytes (Mittelsteadt et al. 2009). However, the *in situ* expression of synaptotagmins and other SNARE-associated proteins, such as Sec1/Munc18-like proteins, in astrocytes still needs to be determined.

Overall, compared with neurons, astrocytes appear to possess a distinct set of operational SNARE proteins for regulating exocytosis *in vivo*. It is known that VAMP isoforms with similar structural properties participate in the formation of several different SNARE complexes by assembling with more than one set of partners (Fasshauer et al. 1999; Fasshauer et al. 1998). Thus, although astrocytes *in situ* and *in vivo* might not express the same SNARE proteins as synaptic terminals (e.g. SNAP25B, and syntaxin 1A), the ternary SNARE fusion complex in astrocytes could also assemble from VAMP2/3 or TI-VAMP/VAMP7, SNAP23, and syntaxin SNARE proteins (Hamilton and Attwell 2010; Montana et al. 2009). A recent study on cultured astrocytes expressing pHluorin-tagged VAMP2 suggests that a limited number, up to five, of such SNARE complexes could be sufficient to conduct single-vesicle fusion in astrocytes (Singh et al. 2014).

Astrocytes contain vesicles that differ in size, protein expression, and gliosis signaling molecule(s) content

Similar to neurons (Klyachko and Jackson 2002), astrocytes contain different types of secretory vesicles carrying various types of chemical signals. In cultured astrocytes, several vesicular compartments undergo regulated exocytosis, including clear electron-lucent SLMVs, which strongly resemble, in morphology, synaptic vesicles (SVs) of nerve terminals (Bergersen and Gundersen 2009; Bezzi et al. 2004; Crippa et al. 2006; Jourdain et al. 2007), dense-core vesicles (DCVs) (Calegari et al. 1999), and secretory lysosomes (Li et al. 2008). These vesicles may (co)store and release low (e.g., 551 Da ATP) and/or high (e.g., approximately 3 kDa ANP) molecular weight chemical transmitters (Guek et al. 2012; Parpura and Zorec 2010).

SLMVs are considered the main storage compartment for low molecular weight transmitters, glutamate (approximately 147 Da) and D-serine (approximately 105 Da), in astrocytes. Glutamate is synthesized by astrocytes *de novo* as a by-product of tricarboxylic

acid (i.e., from α -ketoglutarate, a tricarboxylic acid cycle intermediate) involving the astrocyte-specific enzyme pyruvate carboxylase (Hertz et al. 1999). L-serine is converted to D-serine with astrocytic serine racemase (Martineau et al. 2013). In culture, immunocytochemical studies revealed that astrocytic SLMVs express VGLUTs 1, 2, and 3, which use the H^+ gradient created by V-ATPase to refill vesicles with glutamate (Bezzi et al. 2004; Martineau et al. 2013; Montana et al. 2004; Zhang et al. 2004b). However, the associated chloride flux across the vesicular membrane appears to play an important role in determining the gradient for glutamate uptake in the vesicular lumen (Freneau et al. 2004). Vesicular D-serine transporters were recently functionally identified in astrocytic immunopurified vesicles, although the molecular identity remains elusive at the moment. Similar to VGLUTs, they likely use the H^+ gradient created by V-ATPase to (re)fill the vesicles with D-serine (Martineau et al. 2013). Using electron microscopy with immunogold labeling, VGLUTs 1–2 and D-serine were shown to associate with SLMVs also in astrocytes *in situ*, in rat hippocampal and cerebral cortex brain slices, respectively (Bezzi et al. 2004; Martineau et al. 2013), suggesting that SLMVs *in vivo* may store and release glutamate and D-serine. However, the results of a recent study, where immunolabeling of VGLUTs with various antibodies was used on both wild type and VGLUT1-3 knockout mice, argue against the expression of VGLUTs in SLMVs in cultured astrocytes and *in situ* in the brain grey matter of the primary somatosensory cortex, the thalamic ventrobasal nucleus, hippocampus, and cerebellum (Li et al. 2013). The observed discrepancies between *in situ* preparations might be due to the subcellular, inter-individual astrocyte heterogeneity (Bezzi et al. (2004) observed with only a minority of astrocytes and astrocytic processes expressing VGLUT transcripts and protein *in situ* (Bezzi et al. 2004)), as well as inter-regional heterogeneity in astrocyte properties (Oberheim et al. 2012), or due to different experimental approaches used by different laboratories. SLMVs *in situ* were found in close proximity to the plasma membrane in perisynaptic astrocytic processes and have estimated diameters of 30–100 nm. These vesicles are present in much smaller packs (2–15 vesicles) and are grouped in a non-particular orderly manner than SVs of similar size in nerve terminals, where large pools of SVs exist with hundreds to thousands of SVs per synapse (Bergersen et al. 2012; Bezzi et al. 2004; Jourdain et al. 2007; Martineau et al. 2013). In hippocampal slices, D-serine was shown to be released from much larger vesicles (1–3 μm in diameter), which are likely generated by intracellular fusion of smaller vesicles, other organelles, or both following sustained Ca^{2+} or mechanical stimulation (Kang et al. 2013). Glutamate and D-serine were suggested to be co-stored inside the same SLMV (Martineau et al. 2008) because in cultured astrocytes the SNARE proteins VAMP2 and VAMP3 are colocalized with VGLUTs (Bezzi et al. 2004; Montana et al. 2004) and D-serine (Martineau et al. 2008; Mothet et al. 2005). By contrast, an *in situ* study using immunogold labeling showed that glutamate and D-serine are stored in distinct SLMVs within the same astrocyte (Bergersen et al. 2012). A recent investigation on immunopurified astrocytic vesicles demonstrated that these vesicles can indeed co-store both glutamate and D-serine (Martineau et al. 2013). A comparison of isolated SLMVs from cultured astrocytes (Crippa et al. 2006; Martineau et al. 2013) and isolated neuronal SVs shows that astrocytic SLMVs contain D-serine and glutamate, whereas isolated neuronal SVs contain glutamate, glycine, and GABA but are devoid of D-serine (Martineau et al. 2013; Sild and Van Horn 2013), perhaps indicating distinct physiological roles of SLMVs and SVs in the CNS.

Large dense-core vesicles (LDCVs) are considered the major vesicular compartment for the storage and release of neuropeptides and hormones from neuroendocrine cells (Burgoyne and Morgan 2003) and neurons (Klyachko and Jackson 2002). Astrocytic DCVs exhibit ultrastructure similar to the LDCVs found in neuroendocrine cells and neurons. The DCVs in cultured astrocytes contain the secretory proteins secretogranins II (Calegari et al. 1999; Paco et al. 2009; Prada et al. 2011) and III (Paco et al. 2010). In addition to secretogranins, DCVs in culture also store chromogranins (Hur et al. 2010), ANP (Kreft et al. 2004; Paco et al. 2009), neuropeptide Y (Prada et al. 2011; Ramamoorthy and Whim 2008), and a fraction of cellular ATP (Coco et al. 2003; Pangrsic et al. 2007). DCVs containing secretogranins were recently reported in astrocytes in human brain tissue (Hur et al. 2010), indicating the existence of DCV vesicles in astrocytes *in situ*. DCVs are larger (100–600 nm; (Calegari et al. 1999; Hur et al. 2010; Prada et al. 2011) than SLMVs and do not colocalize with VAMP2 and VGLUT1 in culture, indicating that they belong to a vesicle population distinct from SLMVs (Kreft et al. 2004; Paco et al. 2009; Ramamoorthy and Whim 2008). Cargo molecules in DCVs are discharged from astrocytes upon stimulation; whether they are co-released from the same population of DCVs or belong to distinct populations of DCVs still needs to be investigated. Similar to LDCVs and SVs in neurons, which may both undergo Ca^{2+} -regulated exocytosis in the same nerve terminal (Klyachko and Jackson 2002), DCVs and SLMVs can also co-exist within the same astrocyte (Paco et al. 2009; Ramamoorthy and Whim 2008). DCVs may also serve as inositol-1,4,5-triphosphate (IP_3)-sensitive intracellular Ca^{2+} stores because all three isoforms of IP_3 receptors (IP_3Rs), acting as IP_3 -gated Ca^{2+} channels, were detected on DCV membranes in brain tissue astrocytes (Hur et al. 2010).

In cultured astrocytes, secretory lysosomes store ATP (Jaiswal et al. 2007; Li et al. 2008; Zhang et al. 2007). These secretory organelles are devoid of VGLUTs and VAMP2 (Liu et al. 2011; Zhang et al. 2007), indicating that they belong to a vesicle population distinct from SLMVs. Instead, these astrocytic secretory lysosomes express lysosomal-specific markers, such as cathepsin D and lysosomal-associated membrane protein 1 (LAMP1, also known as cluster of differentiation 107a; (Zhang et al. 2007), Rab 7, SNARE protein TI-VAMP/VAMP7, which contributes to TeNT-independent exocytotic release of ATP (Verderio et al. 2012), and vesicular nucleotide transporter (Sawada et al. 2008), which is involved in storage of ATP (Oya et al. 2013) released from secretory lysosomes in astrocytes (Lalo et al. 2014). These vesicles, 300–500 nm in diameter (Chen et al. 2005; Zhang et al. 2007), can be specifically labeled with dextrans (Jaiswal et al. 2002; Vardjan et al. 2012), recycling FM dyes, and a fluorescent ATP analogue MANT-ATP (Zhang et al. 2007), and co-exist with SLMVs in the same astrocyte (Liu et al. 2011). Whether secretory lysosomes and DCVs are involved in gliosignaling molecule release *in situ* has not yet been thoroughly investigated.

Astrocytes exhibit a G protein-coupled receptor-dependent cytosolic excitability linked to exocytotic release

In contrast to neurons, which exhibit electrical plasmalemmal excitability (firing action potentials) that leads to neurotransmitter release from synaptic terminals, astrocytes are electrically silent and display only cytosolic excitability. The hallmark of cytosolic

excitability is a transient increase in cytosolic levels of Ca^{2+} as a consequence of the complex activation of various receptors, pumps, and transporters that glial cells express on their plasma membrane and endomembrane, most notably that of the smooth ER, which acts as the dominant intracellular Ca^{2+} store (Verkhratsky and Kettenmann 1996). Therefore, as a member of the tripartite synapse, astrocytes use plasma membrane receptors to sense neurotransmitters released from the synapse during synaptic activity. Astrocytes also detect a myriad of other signaling molecules present in the brain parenchyma, some of which are released by astrocytes themselves. Signaling molecules binding to their receptors may increase not only cytosolic levels of Ca^{2+} but also other astrocytic cytosolic signals, such as cyclic adenosine monophosphate (cAMP). Cytosolic excitability may lead to exocytotic release of a variety of molecules from astrocytes, including gliotransmitters and other gliosignaling molecules, which in turn can interact with the receptors on synaptic terminals modulating neuronal excitability (Calegari et al. 1999; Parpura and Verkhratsky 2012) or affect receptors on other neighboring cells in a paracrine or autocrine manner.

Astrocytes express a large number of various types of receptors in culture and *in situ*, and many of these receptors are metabotropic high affinity G protein-coupled receptors (GPCRs) (Agulhon et al. 2008; Parpura and Verkhratsky 2012; Zorec et al. 2012) that only slowly deactivate and desensitize. It has been recently hypothesized that high affinity slowly desensitizing GPCRs likely enable astrocytes to efficiently sense the low amount of neurotransmitters that reaches their perisynaptic processes which are relatively distant from the synaptic cleft (Araque et al. 2014). In general, when a GPCR is activated by its extracellular ligand, a conformational change is induced in the receptor that is transmitted to an attached intracellular heterotrimeric G protein complex. Depending on the type of G protein subunit isoforms, different signals are induced in cells. Activation of the G_q subunit leads to the stimulation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to diacylglycerol (DAG) and IP₃ (**Figure 1**). In astrocytes, binding of IP₃ to IP₃Rs located on the ER ((Hua et al. 2004) or even on secretory vesicles (Hur et al. 2010) increases cytosolic Ca^{2+} levels through the release of Ca^{2+} from these intracellular organelles. The activation of ryanodine receptors on the ER may also increase cytosolic Ca^{2+} levels through the release of Ca^{2+} from the ER (Hua et al. 2004). The Ca^{2+} signal arising from the generation of IP₃ may be amplified by activating further Ca^{2+} release from IP₃Rs and ryanodine receptors in a process called Ca^{2+} -induced Ca^{2+} release (Leybaert and Sanderson 2012). In addition, mitochondria, otherwise acting as metabolic furnaces, have a role in Ca^{2+} buffering in astrocytes through Ca^{2+} uptake and storage (Reyes and Parpura 2008; Simpson and Russell 1998). The Ca^{2+} can also enter astrocytes from the extracellular space through voltage-gated Ca^{2+} channels (Latour et al. 2003; MacVicar 1984; Parri et al. 2001), as observed in culture and *in situ* in tissue slices of the rat ventrobasal thalamus (Parri et al. 2001), but not in hippocampal tissue astrocytes (Carmignoto et al. 1998). The observed discrepancies between the *in situ* data may be due to astrocyte regional heterogeneity or different recording techniques used (optical vs. electrical). Ca^{2+} may also enter into the cytosol through ionotropic receptors (Lalo et al. 2011), and the sodium-calcium exchanger (Reyes et al. 2012) as well as through the transient receptor potential (TRP) A1 channel (Shigetomi et al. 2013; Shigetomi et al. 2012) and TRP canonical 1 channel (Malarkey et al. 2008) that is acting as a conduit for store-operated calcium entry that, along with store-

specific Ca^{2+} -ATPase, replenish the depleted ER store. Despite these myriad potential mechanisms, G_q GPCR activation and release of Ca^{2+} from IP_3 -sensitive internal stores is the best-accepted mechanism for cytosolic Ca^{2+} increases in astrocytes. Unlike G_q GPCR activation, stimulation of G_s GPCR subunits in astrocytes triggers adenylyl cyclase (AC) to catalyze the conversion of ATP to cAMP (Rathbone et al. 1991; Vardjan et al. 2014a). This cAMP activates a number of effectors in the cell, primarily cAMP-dependent protein kinase A, which, by phosphorylating cytoplasmic and nuclear targets, mediates many different functional effects. Signaling via cAMP-activated GTP-exchange protein (de Rooij et al. 1998), cAMP-gated ion channels, and Popeye domain-containing proteins (Froese et al. 2012) may also be present (Beavo and Brunton 2002).

In astrocytes, G_q -induced cytosolic Ca^{2+} increases occur as either oscillations or sustained elevations (Parpura and Verkhratsky 2012; Volterra et al. 2014; Zorec et al. 2012). Ca^{2+} excitability has been observed in culture (Cornell-Bell et al. 1990), in brain slices *in situ* (Pasti et al. 1997), and *in vivo* (Hirase et al. 2004) and may occur spontaneously or in response to neuroligands, including neurotransmitters (Cornell-Bell et al. 1990). Ca^{2+} excitability can propagate from an excited astrocyte to its neighboring unstimulated astrocytes in the form of intercellular Ca^{2+} waves, which are carried by diffusion of IP_3 or Ca^{2+} through gap junctions (Scemes et al. 2000) or via astrocytic release of glutamate and/or ATP and subsequent receptor-mediated activation of neighboring astrocytes (Bowser and Khakh 2007b; Guthrie et al. 1999; Innocenti et al. 2000). These waves travel 10–20 $\mu\text{m}/\text{s}$ (Leybaert and Sanderson 2012). Although the methods for Ca^{2+} imaging have improved in the past years, the range of physiological subcellular Ca^{2+} activity in astrocytes (Ca^{2+} microdomains) *in vivo* may extend beyond what can be detected at present due to highly branched “nanoscopic” architecture of astrocytes. Besides thicker more proximal branches, the individual astrocyte possesses also multiple ultrathin (20–200 nm wide) perisynaptic protrusions that may connect with each other with gap junction(s). However, individual neighboring perisynaptic protrusions cannot be clearly distinguished with current real-time optical techniques, and may be functionally distinct from the thicker branches, where Ca^{2+} recordings can be easily obtained (Rusakov 2015).

By contrast, G_s activation induces persistent cAMP elevations (Vardjan et al. 2014a). Whether G_s -induced cAMP excitability can be propagated between neighboring astrocytes (by intercellular cAMP waves), as was observed for Ca^{2+} excitability, needs to be evaluated. It was suggested that G_q - and G_s -mediated pathways in astrocytes may interact, as activation of the G_s -signaling pathway may potentiate G_q -mediated Ca^{2+} responses (Jiménez et al. 1999; Vardjan and Zorec 2015) and vice versa (Balázs et al. 1998).

Both G_q and G_s GPCR signaling pathways were shown to be involved in exocytotic release of gliotransmitters from astrocytes. It is well established that Ca^{2+} elevation in astrocytes triggers the exocytotic release of glutamate (Bezzi et al. 1998; Bezzi et al. 2004; Parpura et al. 1994; Pasti et al. 2001; Zhang et al. 2004b), ATP (Bal-Price et al. 2002; Coco et al. 2003), secretogranin II (Calegari et al. 1999), ANP (Krzan et al. 2003), and D-serine (Mothet et al. 2005) in culture. By contrast, the roles of cAMP elevations are much less studied in connection to the exocytotic release of gliotransmitters and other gliosignaling molecules from astrocytes. However, it is known that in culture elevations in cAMP can

trigger the exocytotic release of secretogranin II from peptidergic vesicles (Calegari et al. 1999) and possibly ATP from late endolysosomal compartments (Vardjan et al., in preparation). Moreover, enhanced Ca^{2+} -triggered vesicle-based release of ANP was observed in cultured astrocytes pretreated with the membrane permeable cAMP analogue dibutyryl cAMP (Paco et al. 2009). It is currently unknown whether cAMP triggers the fusion of gliosignaling vesicles *de novo* or only modulates the fusion pore dynamics of already pre-fused vesicles by increasing the size and open time of a fusion pore between the vesicle and plasma membranes, as was observed in neuroendocrine cells (Calejo et al. 2013).

Excitation-secretion coupling in astrocytes

The efficiency of secretory cell function is determined by the time required to detect and respond to a stimulus. Various approaches have been used in cultured astrocytes to monitor the temporal dynamics of vesicle-based release at the single-vesicle level, including (1) electrophysiological techniques, such as amperometry (Chen et al. 2005) and membrane capacitance (C_m) measurements using cell-attached patch-clamp recordings (Kreft et al. 2004; Rituper et al. 2013), which were also combined with UV-flash photolysis-induced increases in cytosolic Ca^{2+} levels (Kreft et al. 2004), and (2) optical techniques, such as real-time confocal microscopy and total internal reflection fluorescence microscopy (TIRFM) in combination with fluorescent markers of vesicular cycling/fusion, for example, FM dyes (Li et al. 2008; Liu et al. 2011; Zhang et al. 2007), acridine orange (Bezzi et al. 2004; Domercq et al. 2006), quinacrine (Pangrsic et al. 2007; Pryazhnikov and Khiroug 2008), fluorescent dextrans (Jaiswal et al. 2007), MANT-ATP (Zhang et al. 2007), and genetically encoded chimeric proteins between specific membrane/luminal vesicle markers and green fluorescence protein (GFP)- or mCherry-derived proteins (Marchaland et al. 2008).

Studies with fluorescently tagged VGLUT1/2-containing vesicles (i.e., VGLUT-pHluorin and VGLUT-EGFP, which are chimeric proteins of VGLUT and a pH-sensitive GFP protein (Miesenböck et al. 1998) or EGFP) revealed that fusion events in isolated astrocytes occur within hundreds of milliseconds after the rise of cytosolic Ca^{2+} evoked by activation of either metabotropic glutamatergic receptors (Bezzi et al. 2004; Cali et al. 2008; Marchaland et al. 2008) or purinergic receptors (Santello et al. 2011). Although VGLUT1/2 and VAMP2 are expressed in SLMVs (Bowser and Khakh 2007a; Liu et al. 2011), markedly slower kinetics for vesicular fusion were reported using synaptopHluorin (spH), a chimeric protein between VAMP2 and pHluorin (Bowser and Khakh 2007a; Sankaranarayanan and Ryan 2000). Liu and colleagues (Liu et al. 2011) showed that the Ca^{2+} ionophore ionomycin triggers exocytotic fusion of spH-labeled SLMVs within seconds. Similarly, a TIRFM study (Malarkey and Parpura 2011) showed slow exocytotic bursts occurring within 6 s after mechanical stimulation of astrocytes; however, a variety of other stimuli (ATP, bradykinin, the Ca^{2+} ionophore 4-Br-A23187, α -latrotoxin, or hypertonicity) caused a relatively sustained rate of fusion, lasting for minutes following an apparent delay greater than 1 min. Secretion from neuropeptide Y-positive peptidergic vesicles occurred with a delay greater than 1 min upon glutamate (Ramamoorthy and Whim 2008) or ionomycin stimulation (Prada et al. 2011). Moreover, exocytotic release of fluorescent emerald green-tagged AMP from peptidergic vesicles in 8-Br-cAMP-differentiated astrocytes occurred over a time scale

of minutes following ionomycin application (Paco et al. 2009). When the exocytotic release of lysosomes was studied using astrocytic lysosomes labeled with FM dyes (in contrast to neurons, where FM-dyes solely label SVs upon cell stimulation (Gaffield and Betz 2006), in astrocytes the long-lasting (15 minutes to several hours) exposure to FM-dyes labels mostly lysosomes in a stimulus-independent manner; a shorter incubation time (5 minutes), however, assures preferential loading of FM1-43 into VAMP2/3-laden vesicles as per TeNT sensitivity of the loading procedure (Malarkey et al. 2008)) lysosomes began to fuse with the plasma membrane with a delay greater than 1 min upon calcium ionophore A-23187 (Li et al. 2008), ionomycin, or ATP stimulation (Zhang et al. 2007). The exocytotic fusion of the majority of quinacrine-loaded vesicles that also express lysosomal TI-VAMP (Verderio et al. 2012) occurred with a delay greater than 2 min after exposure to various stimuli, including ionomycin, glutamate, ATP, or UV-induced Ca^{2+} uncaging stimulation (Pangrsic et al. 2007; Pryazhnikov and Khiroug 2008). In addition, the majority of EGFP-LAMP1-labeled and FITC-dextran-labeled lysosomes undergo exocytotic fusion with a delay greater than 40 s upon stimulation with either ionomycin (Liu et al. 2011) or ATP and (R/S)-3,5-dihydroxyphenylglycine, a group I metabotropic glutamate receptor agonist (Jaiswal et al. 2007), respectively.

Although with current experimental techniques gliotransmitter release at a single vesicle level *in situ* and *in vivo* cannot be easily studied and new methodological advances are needed to confirm the above *in vitro* observations, these data strongly indicate that compared with neurons, in which fusion occurs within <0.5 ms upon Ca^{2+} entry to the cytosol to release of neurotransmitters into the synaptic cleft (Neher 2012; Südhof 2012), the exocytotic release of gliosignaling molecules from astrocytes is a much slower process and occurs with a substantial post-stimulus delay. This was also confirmed using C_m measurements on isolated astrocytes, demonstrating that the kinetics of vesicle fusion in astrocytes was at least two orders of magnitude slower than that in neurons (Kreft et al. 2003; Kreft et al. 2004). The delay between the stimulus and the secretory response at the cellular level of an astrocyte is shown in Figure 2.

The relatively slow responsiveness of glial cells may be due to many factors, including the difference in the major source of Ca^{2+} between neurons and astrocytes (the extracellular space vs. the ER, respectively), asynchronous coupling between astroglial Ca^{2+} signals and vesicle fusion (Li et al. 2008; Pryazhnikov and Khiroug 2008), the slow delivery of vesicles to the plasma membrane fusion sites (Potokar et al. 2013), or slower dynamics of molecular mechanisms governing the merger between the vesicular and plasma membranes (Montana et al. 2009). It is likely that the mechanism regulating the merger of the membranes plays an important role because the fast delivery of the Ca^{2+} signal demonstrated using the UV caged photolysis approach revealed a robust heterogeneity of delays between the stimulus and secretory responses among various neuronal, endocrine, and glial cells, with glial cells displaying the longest delay (Table 1), indicating loose excitation-secretion coupling in astrocytes.

Astrocytes exhibit two modes of exocytosis

Exocytotic fusion in cultured astrocytes appears to take two forms, as was observed in neurons and neuroendocrine cells (Harata et al. 2006). Chen et al. (Chen et al. 2005) used amperometry techniques to propose that dopamine-loaded astrocytic vesicles fuse with the plasma membrane by transient (kiss-and-run) exocytosis, with vesicle contents only partially released, or by full-fusion exocytosis. The existence of these two modes of exocytosis was later confirmed using optical studies in which the exocytosis of SLMVs expressing spH (Bowser and Khakh 2007a; Malarkey and Parpura 2011) or SLMVs co-expressing VGLUT1-mCherry/VGLUT1-pHluorin (Marchaland et al. 2008) was studied with TIRF microscopy. In these studies, both modes of SLMV exocytosis were shown to occur simultaneously in the same astrocyte under spontaneous or stimulated conditions. Among the spontaneous exocytotic events, 50–60% were full-fusion events, whereas 40–50% underwent transient-fusion exocytosis. Depending on the stimulus type, the percentage of either type of event shifted toward transient or full-fusion modes of exocytosis upon stimulation, indicating stimulus-dependent regulation of fusion pore opening (Bowser and Khakh 2007a; Chen et al. 2005; Malarkey and Parpura 2011). When the recycling of SLMVs was studied by loading FM 4-64 dye into VGLUT2-EGFP-expressing vesicles, two modes of SLMV recycling were proposed: local recycling (65% of all vesicles), with vesicles remaining in the proximity of the plasma membrane (kiss-and-run/kiss-and-stay recycling), and deeper intra-cytoplasmic recycling (the remaining 35% of vesicles), with vesicles found deep in the cytoplasm (clathrin-mediated endocytosis/bulk retrieval; Cali et al. 2008). Similar to SLMVs, both transient and full-fusion modes of exocytosis have been observed in lysosomes, albeit with dichotomous results reported in various studies. Namely, in mechanically stimulated astrocytes, a rapid and total release of an FM dye was observed, followed by a slower and complete loss of EGFP-sialin (a lysosomal sialic acid transporter) signal (presumably due to diffusion of sialin within the plasma membrane) from the same lysosomes, suggesting that lysosomal fusion in astrocytes completes within seconds, without evidence for kiss-and-run (Li et al. 2008). However, Zhang et al. (2007) reported that the release of FM dyes and MANT-ATP from LAMP1-positive lysosomes stimulated with glutamate or ATP was only partial, implying the transient kiss-and-run mode of lysosomal exocytosis (Zhang et al. 2007). Consistent with this report, recent studies showed that vesicular nucleotide transporter tagged with mCherry remains associated with the lysosomal membrane during release of cathepsin D-Venus from the same lysosomes upon various stimuli (ATP, L-glutamate, and calcium ionophore A23187), suggesting that lysosomes in astrocytes may not fully collapse/fuse to the plasma membrane (Oya et al. 2013). One plausible explanation for these seemingly desperate findings may be the use of different secretagogues as well as means for visualizing the secretion in these studies. The transition between transient and full-fusion exocytosis may depend on vesicle size in endocrine cells (Flaşker et al. 2013), and this needs to be studied in astrocytes to determine whether vesicles with different diameters exhibit different capacities to discharge their cargo, due to distinct fusion pore properties. The existence of both forms of exocytosis in astrocytes *in vivo* and their functional relevance in the CNS have not been determined yet due to the lack of *in vivo* single vesicle imaging methods.

Concluding remarks

In vivo, astrocytes receive input and respond to the activity of surrounding cells (neurons, microglia, oligodendrocytes, and vascular endothelial cells), appearing to actively partner in CNS intercellular signaling. Astrocytes participate in a wide range of secretory processes using vesicle-laden gliosignaling molecules. Loose excitation-secretion coupling supports astrocytic participation in signaling at a slower, modulatory pace than that of the fast synaptic transmission occurring between neurons. Nonetheless, a plethora of evidence supports the existence of regulated exocytosis in astrocytes in culture and the essential components of GPCR-coupled Ca^{2+} signaling and exocytosis were detected in astrocytes *in situ*. Although the physiological relevance of regulated vesicular release of gliosignals from astrocytes *in vivo* and its importance in normal brain information processing is still under debate, this release likely plays a role in modulating neuronal activity during physiological and pathological conditions.

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Main points

- In comparison to neurons, vesicle-based secretion of gliosignaling molecules from astrocytes occurs with a delay to the stimulus.
- This property places astrocytes as signal integrators responding to slow-based signaling processes in the brain.

Figure 1. Sources of Ca²⁺ for regulated vesicle-based secretion from astrocytes

The accumulation of Ca²⁺ in the cytosol may occur (1) following the entry of Ca²⁺ from the extracellular space (ECS) through L-type voltage-gated channels (VGCC), store-operated Ca²⁺ entry (SOCE) via transient receptor potential canonical type 1-containing channels, and the plasma membrane Na⁺/Ca²⁺ exchanger (NCX), and (2) via G protein-coupled receptor (GPCR) activation, which can generate second messengers cAMP, inositol 1,4,5 triphosphate (IP₃), and diacylglycerol (DAG). The GPCR activation in astrocytes retrieves Ca²⁺ from the ER internal stores that possess IP₃ receptors (IP₃R) as well as from ryanodine (Ry)-sensitive channels acting as conduits for Ca²⁺ delivery to the cytosol. The ER store is (re)filled by Ca²⁺-ATPase (i.e., SERCA pumps), which can be blocked by thapsigargin (Thaps). Cytosolic Ca²⁺ levels are modulated by mitochondria. These organelles take up Ca²⁺ via the Ca²⁺ uniporter, which is blocked by ruthenium 360 (Ru360), during the cytosolic Ca²⁺ increase. As cytosolic Ca²⁺ decreases due to the extrusion mechanisms, Ca²⁺ is slowly released by mitochondria into the cytosol via the mitochondrial Na⁺/Ca²⁺ exchanger as well as by the transient opening of the mitochondrial permeability transition pore. This transient opening is indirectly blocked by cyclosporin A (CsA), which binds cyclophilin D (not shown). The increase in cytosolic Ca²⁺ levels is sufficient and necessary to cause the fusion of secretory vesicles (which themselves can act as IP₃-sensitive stores for Ca²⁺) with the plasma membrane, mediating the exit of gliosignaling molecules (such as amino acids, peptides, and ATP) from the vesicle lumen into the ECS. The cAMP-mediated modulation of Ca²⁺ homeostasis may occur at the level of Ca²⁺ entry or extrusion from the cytosol. Moreover, cAMP-mediated mechanisms may directly affect the fusion pore and the extrusion of gliosignals from the vesicle lumen. Drawing is not to scale.

Figure 2. Delay between the increase in cytosolic Ca^{2+} and the increase in membrane capacitance reported for vesicle-based secretory activity in astrocytes
 (A) Typical responses of membrane capacitance (C_m) in three individual astrocytes elicited by flash (UV) photolysis-induced (upward-pointing arrow) elevations in the intracellular calcium concentration ($[Ca^{2+}]_i$). Numbers adjacent to traces indicate measured peak values of $[Ca^{2+}]_i$. (B) Time derivative of responses corresponding to traces in (A) as indicated by the arrows. Dashed vertical lines denote the delays between the flash delivery and the maximal rate of C_m increase. (C) Ca^{2+} dependence of the maximal rate of capacitance increase. The curve was obtained by fitting the data to the following function: $(f \text{ F/s}) = (3008 \pm 792) \times ([Ca^{2+}]^{(5.3 \pm 2.3)}) / ((21.9 \pm 3.8 \mu M)^{(5.3 \pm 2.3)} + [Ca^{2+}]^{(5.3 \pm 2.3)})$. Each filled circle represents the average $\pm$ standard error of three to five measurements. (D) Ca^{2+} dependence of the delay to the maximal rate of capacitance increase. The curve was obtained by fitting the data to the following exponential regression algorithm: $\text{delay (s)} = (0.55 \pm 0.15 \text{ s}) \times (\exp(-(0.057 \pm 0.015 \mu M^{-1}) \times [Ca^{2+}])))$, where the parameters are in the format mean $\pm$ standard error. Modified with permission from Figure 2 in Kreft et al. (2004).

Table 1

Excitation-secretion coupling delay in various types of secretory cells

Cell type	Excitation-secretion delay	Reference
Astrocytes	250 ms	Kreft et al. (2004) <i>Glia</i> . 46:437-445.
Endocrine pituitary cells	25 ms (time constant)	Rupnik et al. (2000) <i>Proc Natl Acad Sci USA</i> . 97:5627-5632.
Chromaffin cells	3 ms	Voets (2000) <i>Neuron</i> . 28(2):537-545.
Rod photoreceptors	3 ms (time constant) 6 ms (time constant)	Kreft et al. (2003) <i>J Neurophysiol</i> . 90:218-25. Thoreson et al. (2004) <i>Neuron</i> . 42:595-605.
Bipolar neurons	1 ms	Heidelberger et al. (1994) <i>Nature</i> . 371:513-515.
Calyx of Held neurons	0.3–1 ms	Bollmann et al. (2000) <i>Science</i> . 289:953-957. Schneggenburger & Neher (2000) <i>Nature</i> . 406:889-893.

Excitation-secretion coupling in various secretory cell types was reported as the delay between the flash photolysis-induced cytosolic $[Ca^{2+}]$ increase (stimulus) and an increase in whole-cell membrane capacitance or as a time constant of the rapid phase of the membrane capacitance response. Membrane capacitance increases indicate vesicular fusions to the plasmalemma.