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ORIGINAL PAPER

Excitable Astrocytes: Ca^{2+}- and cAMP-Regulated Exocytosis

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Abstract During neural activity, neurotransmitters released at synapses reach neighbouring cells, such as astrocytes. These get excited via numerous mechanisms, including the G protein coupled receptors that regulate the cytosolic concentration of second messengers, such as Ca^{2+} and cAMP. The stimulation of these pathways leads to feedback modulation of neuronal activity and the activity of other cells by the release of diverse substances, gliosignals that include classical neurotransmitters such as glutamate, ATP, or neuropeptides. Gliosignal molecules are released from astrocytes through several distinct molecular mechanisms, for example, by diffusion through membrane channels, by translocation via plasmalemmal transporters, or by vesicular exocytosis. Vesicular release regulated by a stimulus-mediated increase in cytosolic second messengers involves a SNARE-dependent merger of the vesicle membrane with the plasmalemma. The coupling between the stimulus and vesicular secretion of gliosignals in astrocytes is not as tight as in neurones. This is considered an adaptation to regulate homeostatic processes in a slow time domain as is the case in the endocrine system (slower than the nervous system), hence glial functions constitute the gliocrine system. This article provides an overview of the mechanisms of excitability, involving Ca^{2+} and cAMP, where the former mediates phasic signalling and the latter tonic signalling. The molecular, anatomic, and physiologic properties of the vesicular apparatus mediating the release of gliosignals is presented.

Keywords Regulated exocytosis · cAMP · Cytosolic Ca^{2+} · Tonic and phasic signalling

Introduction The Gliocrine System

Signal processing in the brain is no longer an exclusive property of neurones, but is shared by astrocytes [1, 2], the most heterogeneous and abundant glial cell type in the central nervous system (CNS). Although recently debated [3, 4], the role of astrocytes in signalling is best appreciated by considering them as a partner of the tripartite synapse, a term coined 15 years ago to highlight gliotransmission [5, 6]. This form of astrocytic signalling, alluding to the relatively fast neuronal signal processing, may, however, be too narrow. There are CNS functions linked to glia that occur in very long time domains, including neurodevelopment, memory maintenance, neuroprotection, homeostatic metabolic mechanisms, all involving vesicle-based signalling that is as slow as the availability of metabolic precursors [7]. Therefore, to accommodate the multitude of glial time domain modes of communication, a more appropriate term than gliotransmission would be the gliocrine system. This is taken by analogy with the endocrine system, which provides homeostatic control of bodily functions in a slower time scale versus the rapid responses of the nervous system.

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Non-neuronal cells, which include astrocytes, exceed the number of neurones in the cerebral cortex that represents 82 % of the total brain mass [8], and are closely associated with neuronal perisynaptic processes. Due to their close association with the synapses, astrocytes influence back neuronal communication after the detection of signals in the environment by their plasma membrane receptors and release of their own signalling molecules (gliosignals): modulators and gliotransmitters [9–11], perhaps also via membrane-bound extracellular vesicles [12]. A single astrocyte may be associated with a large number of neurones. It has been estimated that, in the hippocampal CA1 area in adult rats, there are ~213 synapses/100 μm^3 [13]. Since the estimated volume of a rat astrocyte is ~66,000 μm^3, a single astrocyte in the rat hippocampus can be in association with up to 140,000 synapses [14]. Human hippocampal astrocytes are larger and more complex; their volume is 27 times greater than that of their rodent counterparts, thus a single human astrocyte can be in association with up to 2 million synapses [15], suggesting that their role has expanded with evolution [16]. These synapses are also linked with neurones that have their somata far away, such as those in the locus coeruleus (LC), a primary source of norepinephrine (NA), innervating most brain structures, including the neocortex and hippocampus [17].

When astrocytes are exposed to the extracellular signalling molecules that appear in the extrasynaptic space, they get excited. They typically respond with an increase in the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_i$) due to activation of plasmalemmal G protein coupled receptors (GPCRs). However, some signalling molecules such as NA, released from LC neurones, which act on adrenergic receptors (ARs), a type of GPCR expressed on neurones, microglia, and astrocytes throughout the brain, trigger both intracellular Ca^{2+} and cAMP signalling pathways. GPCR-mediated intracellular signalling in astrocytes has led to the discovery of gliotransmission-based modulation of synaptic transmission [18, 19] and formulation of the concept that three elements, pre- and post-synaptic neuronal along with glial processes, constitute a synapse [20], whose structural–functional partnership has been named the tripartite synapse [5].

Compared with the well-characterized vesicular exocytosis in neurones and neuroendocrine cells, the release mechanisms of gliotransmitters and chemical modulators (collectively termed gliosignal molecules) from astrocytes are still under debate [21–24]. Non-vesicle mechanisms of gliosignal molecule release have been reported such as (1) release via swelling-induced opening of volume-regulated anion channels, connexons/pannexons (hemichannels) (2) ionotropic pore forming $P2X_7$ purinergic receptors, (3) transporters such as reverse uptake by plasma membrane excitatory amino acid (glutamate) transporters, by exchange via the cystine-glutamate antiporter or organic anion transporters (reviewed in [25]), (4) the two-pore-domain potassium (K2P) channel, Trek-1, and the Bestrophin-1 (Best-1) channel [26]. These release mechanisms are mainly Ca^{2+} independent and appear activated predominantly under pathologic conditions [22, 27]. However, astrocytes were shown to release gliosignal chemical messengers in vitro and in situ by a Ca^{2+}-dependent vesicle-based mechanism (i.e. exocytosis), by using a variety of experimental approaches, including optical methods, membrane capacitance measurements, electrochemical amperometry, as well as via selective interference with proteins of the exocytotic machinery [28, 29]. The process of exocytosis, which may occur under physiologic conditions, involves the merger of gliosignal-containing vesicles with the plasma membrane.

This review addresses the excitability mechanisms involving GPCRs in astrocytes and the vesicular release mechanisms of gliosignals, such as amino acids (glutamate [21, 29, 30], D-serine [31, 32]), peptides (atrial natriuretic peptide (ANP) [33], secretogranin II and chromogranin [34]), nucleotides (adenosine 5′-triphosphate (ATP) [35–38]) and molecules required for facultative antigen presentation by astrocytes, major histocompatibility class II molecules (major histocompatibility complex (MHC) II complexes [39]). First, we briefly discuss the mechanisms of excitability, then we focus on the molecular machinery underlying gliosignal exocytosis by dealing with different vesicle types in astrocytes. Finally, the vesicle kinetics in gliosignal exocytosis at the single vesicle level is discussed to highlight the relative slowness of this process versus neuronal vesicle communication. The slowness of gliosignal function justifies the notion that astrocytes are an important part of the glyocrine system maintaining homeostasis in the CNS.

GPCR-Mediated Astrocytic Excitability

In contrast to neurones, which exhibit electrical excitability (firing action potentials, transient changes in transmembrane potential) that leads to Ca^{2+}-based neurotransmitter release, astrocytes are electrically silent and display only cytosolic excitability [11]. Astrocytes as part of the tripartite synapse can sense neuronal activity via their plasma membrane receptors to detect neurotransmitters released during synaptic activity. This may increase the cytosolic levels of secondary signalling messengers in astrocytes, such as Ca^{2+} and cyclic adenosine monophosphate (cAMP). Cytosolic excitability may control the exocytotic release of gliosignals from astrocytes that can, in turn, interact with the receptors on the adjacent synaptic terminals

modulating neuronal excitability [34, 40], and likely the activity of other neuronal cell types at more distant locales than the astrocytic gliosignal sources.

To detect chemical signals in their surroundings, astrocytes express a large number of different types of receptors in culture and in situ; many of these receptors are metabotropic high-affinity GPCRs [11, 27, 40]. 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 can be induced in cells. Activation of G_q subunits leads to stimulation of phospholipase C (PLC), which breaks down phosphoinositol diphosphate (PIP2) into diacylglycerol (DAG) and inositol-1,4,5-triphospate (IP3). In astrocytes, binding of IP3 to IP3 receptors (IP3Rs) on the endoplasmic reticulum (ER) [41] or possibly secretory vesicles [42] increases $[Ca^{2+}]_i$ through the release of Ca^{2+} from these intracellular organelles. In addition, activation of ryanodine receptors (RyRs) on the ER may increase Ca^{2+} levels through the release of Ca^{2+} from the ER [41]. The Ca^{2+} signal arising from the generation of IP3 may be amplified by activating further Ca^{2+} release from IP3Rs and RyRs in the process of Ca^{2+}-induced Ca^{2+} release (CICR) [43]. Mitochondria also have a role in Ca^{2+} buffering in astrocytes through Ca^{2+} uptake and storage [44, 45]. Ca^{2+} can also enter astrocytes from the extracellular space through voltage-gated Ca^{2+} channels (VGCCs) [46–48] and ionotropic receptors [49], although G_q GPCR activation and release of Ca^{2+} from IP3-sensitive internal stores is the most accepted mechanism for increases in Ca^{2+} in astrocytes. On the contrary, activation of G_s subunits in astrocytes stimulates the enzyme adenylyl cyclase (AC), which catalyzes the conversion of ATP into cAMP [50, 51]. cAMP may activate a number of effectors in the cell, primarily the cAMP-dependent protein kinase (PKA) which, by phosphorylating cytoplasmic and nuclear targets, mediates many different functional effects, although signalling via cAMP-activated GTP-exchange protein (Epac) [52], cAMP-gated ion channels, and popeye domain containing proteins [53] can also be present [54].

In astrocytes, G_q-induced increases in $[Ca^{2+}]_i$ in astrocytes can be either oscillations or long-duration transient Ca^{2+} spikes [11, 40, 55] (Fig. 1a). They have been observed in culture [56], in brain slices in situ [57], and in vivo [58] and may occur spontaneously or in response to neurotransmitters [56]. Ca^{2+} excitability can be propagated to neighbouring unstimulated astrocytes (intercellular Ca^{2+} waves) by diffusion of IP3 and/or Ca^{2+} via gap junction communication [59] or via astrocytic release of glutamate or ATP into the extracellular space and the subsequent receptor-mediated activation of neighbouring astrocytes

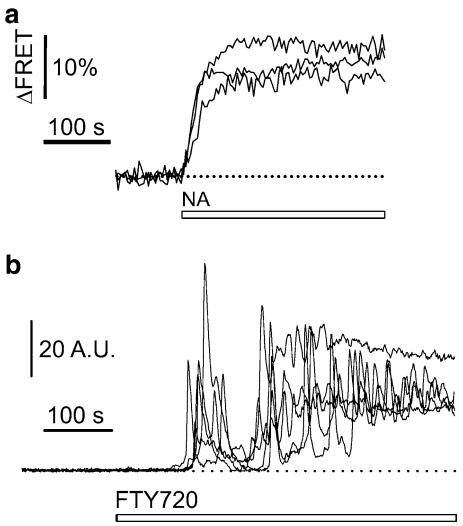

Fig. 1 **a** Norepinephrine (NA) persistently increases intracellular cAMP levels in astrocytes. Representative time-courses of the Epac1-camps (*i.e.* FRET-based cAMP nanosensor) emission ratio from 3 cells after the addition of 1 μM NA. Changes in FRET are expressed as percentages relative to the initial values. **b** The application of fingolimod (FTY720) evokes prolonged transient increases (oscillations). Superimposed time-resolved fluorescence intensity obtained in 5 cells treated with FTY720 (*white bar*). The *thin dotted line* indicates the zero fluorescence level (F_0). Modified with permission from Potokar et al. [123]

[60]. These waves can propagate at ~10–20 μm/s [43]. In contrast to Ca^{2+} oscillations, G_s activation induces a persistent increase in $[cAMP]_i$ [51] (Fig. 1b). Whether G_s-induced cAMP excitability can be propagated between neighbouring astrocytes (intercellular cAMP waves), as has been observed for Ca^{2+} excitability [59], needs to be evaluated. It has been suggested that G_q- and G_s-mediated pathways in astrocytes interact, since activation of the G_s signalling pathway may potentiate G_q-mediated Ca^{2+} responses [61] and vice versa [62]. What appears clear is that although the second messenger Ca^{2+} in astrocytes responds in a more rapid phasic manner (oscillations), so far signalling with the second messenger cAMP appears to exclusively mediate slow tonic (persistent) signals. Thus, if these two systems interact when they modulate downstream effectors, then it is likely that cAMP-mediated effects will have slow temporal characteristics. How these two pathways integrate in regulating vesicular mechanisms is an open question to be addressed in the future.

Both G_q and G_s GPCR signalling pathways have been shown to be involved in exocytotic release of gliosignals from astrocytes. It is well established that increases in Ca^{2+} in astrocytes can trigger exocytotic release of glutamate [21, 29, 30, 63, 64], ATP [35, 36], secretogranin II [34], ANP [33], and D-serine [32]. On the contrary, increases in cAMP in astrocytes in connection with exocytotic gliosignal release are much less studied; however cAMP

can trigger exocytotic release of secretogranine II from peptidergic vesicles [34] and ATP from late endolysosomes. Moreover, enhanced Ca^{2+}-triggered exocytosis of ANP has been observed in astrocytes pretreated with the membrane-permeable cAMP analogue dibutyryl cAMP [65]. Whether cAMP triggers the fusion of gliosignal 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 the plasma membrane, as has been observed in neuroendocrine cells [66], is not known.

Proteins Necessary for SNARE-Dependent Gliosignal Vesicular Release

In neurones, signalling involves propagation of the action potential down the axon to the synaptic terminal, where synaptic vesicles are docked and get fused with the plasma membrane. In this process, a key function is played by the SNARE (soluble *N*-ethylmaleimide-sensitive-factor attachment protein receptor) proteins (i.e. synaptosome-associated protein of 25 kDa [SNAP25], syntaxin, vesicle-associated membrane protein 2 [VAMP2]), localized at the active zones, structurally organized vesicle release zones, at the nerve terminal [67]. Electron microscopy studies have shown that astrocytes lack active zones, which can be seen in presynaptic neurons [21, 68]. However, like neurons, astrocytes do express SNARE proteins and SNARE-associated proteins such as synaptotagmins and SM (Sec1/Munc18-like) proteins (review in [69]), although the SNARE components of the exocytotic apparatus between astrocytes and neurones are not identical.

The first evidence of the presence of SNARE complex in astrocytes was shown by immunocytochemistry studies on cultured astrocytes [70]. Further data obtained on cultured and freshly isolated astrocytes showed that astrocytes express a variety of SNARE proteins; R-SNARE (vesicular SNARE) proteins synaptobrevin 2/VAMP2, cellubrevin/VAMP3 [31, 32, 70–73], tetanus neurotoxin-insensitive VAMP (TI-VAMP)/VAMP7 [74], and QSNARE (target membrane SNARE) proteins; synaptosome-associated protein of 23 kDa (SNAP23), SNAP25, and syntaxins (STX) 1, 2, 3 and 4 [30, 65, 75]. Besides SNARE proteins, cultured astrocytes also express SNARE-associated proteins such as synaptotagmin 4 [76]. Although this protein does not exhibit significant Ca^{2+}-binding properties, it belongs to the family of proteins taking part in regulated Ca^{2+}-evoked exocytosis [77]. Astrocytes also express the isoforms of Munc18 [65], which interact with the SNARE complex. The functional cleavage of SNARE proteins with *Clostridium*, tetanus and various types of botulinum neurotoxins reduces glutamate release in cultured astrocytes as well as exocytosis as measured by the attenuation of membrane capacitance (C_m) increase [19] and a reduction in amperometric spikes [78], further indicating that astrocytes in culture possess proteins necessary for regulated exocytosis. Moreover, SNARE proteins VAMP2 and VAMP3 co-localize with ATP [72] or D-serine [31] storing vesicles in cultured astrocytes.

Cultured astrocytes are considered to be different in many respects from astrocytes in situ (in the surrounding environment, morphology, protein expression, etc.) and may differ in the expression of SNARE proteins. In situ studies using immunogold cytochemistry and confocal microscopy have confirmed the presence of the SNARE proteins VAMP2 [73], VAMP3 [21, 30, 68, 79, 80], TI-VAMP/VAMP7 [74], SNAP23 [80], and syntaxin 1 [80] in tissue astrocytes. VAMP3 was shown to co-localize with the vesicular glutamate transporters VGLUT1 and 2 on small synaptic-like microvesicles (SLMVs) that store gliotransmitter glutamate [21, 30, 68, 79] and TI-VAMP/VAMP7 colocalizes with the markers of late endolysosomal compartments [74] that store gliosignal molecule ATP [35–38]. Inactivation of VAMP2 and/or VAMP3 in astrocytes by tetanus neurotoxin abolishes the release of glutamate or D-serine from astrocytes in situ [68, 81, 82], implying SNARE-mediated release of these chemical messengers (gliosignals). In addition, a mouse model that allows expression of a dominant negative (dn) SNARE transgene in astrocytes was generated to interfere specifically with VAMP2 and 3 in astrocytes [83, 84]. Use of these mice has revealed that synaptic properties change [84–88] suggesting the involvement of astrocytic VAMP2/3-dependent exocytosis in these processes. mRNA studies in the tissue have detected co-expression of several synaptotagmin isoforms in astrocytes, including synaptotagmins 7 and 11, synaptotagmins 1 and 4 [89], although in situ the expression of synaptotagmins and other SNARE-associated proteins, such as SM proteins in astrocytes, still needs to be determined.

Recently, the spatial arrangement of VAMP2 in a single astrocytic vesicle was examined by super-resolution microscopy and by labelling luminal and extravesicular domains of VAMP2 in cultured astrocytes. The VAMP2 protein was tagged at the C- and N-termini with a pair of fluorophores, which allowed measurements of VAMP2 length and spatial arrangement down to 20 nm resolution in cultured astrocytes [90]. To assess the functional significance of VAMP2 in vesicle fusion, the pH sensitive yellow synaptopHluorin (YpH; in which fluorescence increases with pH) was targeted to the vesicle lumen by attaching it to the C terminus of VAMP2, while the N terminus of the same protein expressed in astrocytes was marked with anti-VAMP2 antibody. The results revealed that a single astrocytic vesicle is laden by <25 molecules of

VAMP2, a subset of which, ~5 VAMP2 molecules, are necessary for single vesicle fusion [90], a much lower number than that previously determined in single synaptic vesicles [91]. It is tempting to speculate that such a reduced number of VAMP2 molecules may be related to the different stability and molecular properties of the SNARE complex characteristic of astrocytes [92].

Although the presence of some typical neuronal SNARE and SNARE-associated proteins (such as SNAP25, synaptotagmin-1 and -2 and synaptophysin [73]) in astrocytes in situ has not been confirmed, these data strongly imply the existence of an operational SNARE protein complex for regulated exocytosis in astrocytes in vivo. It is known that VAMP isoforms with similar structural properties can participate in the formation of several different SNARE complexes by assembling with more than one set of partners [93, 94]. Thus, although astrocytes in situ do not express the same SNARE proteins as synaptic terminals (VAMP2, SNAP25, and syntaxin) the ternary SNARE fusion complex in astrocytes might assemble from VAMP2/3 or TI-VAMP/VAMP7, SNAP23, and syntaxin SNARE proteins [22].

Astrocytic Vesicles Differ in Radius, Protein Expression, and Gliosignal Content

Astrocytes, as neurones [95], contain different types of vesicular organelles carrying various types of gliosignal transmitters. In cultured astrocytes, several vesicular compartments were determined that may undergo regulated exocytosis, including clear electron-lucent small SLMVs; their morphology strongly resembles that of synaptic vesicles (SVs) of nerve terminals [21, 68, 71, 79], dense core vesicles (DCVs)/less DCVs [34, 96], and secretory lysosomes [24]. These vesicles may (co)store and release low and/or high molecular weight chemical messengers [28, 96].

SLMVs are the main storage compartment for low molecular weight gliosignal transmitters, glutamate and D-serine, in astrocytes. Glutamate can be synthesized by astrocytes de novo as a by-product of tricarboxylic acid (TCA), i.e. from α-ketoglutarate, a TCA intermediate, involving the astrocyte-specific enzyme pyruvate carboxylase [97]. L-serine can be converted to D-serine with astrocytic serine racemase [98]. In culture, astrocytic SLMVs express vesicular glutamate transporters (vGLUTs 1 and 2), which use the H^+-gradient created by vATPase to refill vesicles with glutamate [21, 30, 98]. Vesicular D-serine transporters (VSERTs) have recently been identified in astrocytic immunopurified vesicles and, similar to vGLUTs, they likely use the H^+-gradient created by ATPase to refill serine in the vesicles [98]. Although a recent study argues against expression of vGLUTs in immunostained brain astrocytes [23], VGLUTs 1–3 and D-serine were also shown to be associated with SLMVs in in situ studies. SLMVs were found in close proximity to the plasma membrane in perisynaptic processes of astrocytes and have estimated diameters of 30–100 nm. They are present in much smaller numbers (2–15 vesicles) and less ordered groups than SVs of similar size in nerve terminals, where large pools of SVs exist with 100–1000s SVs per synapse [21, 68, 98, 99]. In hippocampal slices, D-serine was shown to be released from much larger vesicles (1–3 μm in diameter) that are likely generated by intracellular fusion of SLMVs and/or other organelles following sustained increases in $[Ca^{2+}]_i$ or mechanical stimulation [100]. Glutamate and D-serine were suggested to be co-stored inside the same SLMV [31], since the SNARE protein VAMP2 is colocalized with both vGLUTs [21] and D-serine [31, 32] in cultured astrocytes. Although an in situ study using immunogold labelling has shown that glutamate and D-serine are stored in a distinct SLMV population within the same astrocyte [99], a recent investigation on immunopurified astrocytic vesicles demonstrated that these vesicles can indeed co-store both gliosignal transmitters [98]. Comparison of isolated SLMVs from culture astrocytes [71, 98] and isolated neuronal SVs shows that astrocytic SLMVs contain D-serine and glutamate, whereas isolated SVs contain glutamate, glycine and GABA but are devoid of D-serine [98, 101], indicating a distinct physiological role of SLMVs and SVs in the CNS.

Large dense core vesicles (LDCVs) are considered to be the major vesicular compartment for the storage and release of neuropeptides and hormones in neuroendocrine cells [102] and neurones [95]. Astrocytic DCVs are very few in number and exhibit an ultrastructure similar to the LDCVs found in neuroendocrine cells and neurones. DCVs in cultured astrocytes contain the secretory proteins secretogranins II [34, 65, 103] and III [104]. Besides secretogranins, DCVs/less DCVs [28] in culture can also store chromogranins [42], ANP [19, 65], neuropeptide Y (NPY) [103, 105] and a fraction of cellular ATP [36, 37]. DCVs containing secretogranins were recently reported in astrocytes in human brain tissue [42], confirming the existence of DCV vesicles also in situ. DCVs are typically larger than SLMVs (~100–600 nm) [34, 42, 103] and do not apparently co-localize with VAMP2 and vGLUT1 in culture, indicating that they belong to a distinct vesicle population like SLMVs [65, 105]. DCV gliosignal molecules are discharged from astrocytes upon stimulation; whether they are co-released from the same DCVs or belong to distinct subpopulations of DCVs still needs to be investigated. Similarly, like LDCVs and SVs in neurons, where both vesicle types may undergo Ca^{2+}-regulated exocytosis in the same nerve terminal [95], DCVs and SLMVs can also

co-exist within the same astrocyte [65, 105]. DCVs may also represent the IP3-sensitive intracellular Ca^{2+} stores, since all three isoforms of IP3 receptors (IP3Rs, IP3-gated Ca^{2+} channels) have been detected on the DCV membranes in brain tissue astrocytes [42].

In cultured astrocytes, secretory lysosomes are the major storage compartment of vesicular ATP [24, 38, 106]. These vesicles are devoid of vGLUTs and VAMP2 [38, 107], indicating that they belong to a distinct vesicle population as SLMVs. Instead they express lysosomal specific markers such as cathepsin D and LAMP1 [38], Rab 7, SNARE protein TI-VAMP/VAMP7, which contributes to tetanus toxin independent exocytosis of ATP [74], and vesicular nucleotide transporter (VNUT) [108], which is involved in ATP storage [109] and release [86] from secretory lysosomes in astrocytes. These vesicles can be specifically labelled with dextrans [39, 110], FM dyes, MANT-ATP [38] and exhibit diameters of 300–500 nm [38, 78, 90] and can co-exist with SLMVs in the same astrocyte [107].

Whether DCVs and secretory lysosomes are involved in gliosignal transmitter release in situ has not been thoroughly investigated yet, although quantal events of ATP have been shown in cultured and in in situ astrocytes [37, 86].

Slow Vesicle-Mediated Gliosignal Release from Astrocytes

To monitor the temporal dynamics of gliosignal (gliotransmitter) release at the single vesicle level, various techniques have been used on astrocytes in culture, including (1) electrophysiologic techniques such as amperometry [78] and membrane capacitance (C_m) measurements using cell-attached patch-clamp recordings [19, 111] and (2) optical techniques such as real-time confocal microscopy and total internal reflector fluorescence microscopy (TIRFM) in combination with fluorescence markers, e.g. FM-styryl dyes [24, 38, 107], acridine orange [21, 112], quinacrine [38, 113], fluorescent dextrans [106], the ATP analogue MANT-ATP [38], and genetically encoded chimeric proteins between specific membrane/luminal vesicle markers and green fluorescence protein (GFP)- or mCherry-derived proteins [114].

Studies with fluorescently tagged vGLUT-containing vesicles (i.e. vGLUT-pHluorins, and vGLUT-EGFPs, chimeric proteins between vGLUT and a pH sensitive GFP protein [115] or EGFP), revealed that fusion events in isolated astrocytes occur within hundreds of milliseconds after the increase in Ca^{2+} evoked by either activation of metabotropic glutamatergic receptors [21, 114, 116] or purinergic receptor activation [117]. Although vGLUT and VAMP2/3 are both expressed in SLMVs [118, 107], different much slower kinetics of vesicular release have been reported by using fluorescently tagged VAMP2 and synaptopHluorin (spH), a chimeric protein between VAMP2 and pHluorin [118, 119]. Liu et al. [107] showed that the Ca^{2+} ionophore ionomycin triggers exocytosis of spH-labelled SLMVs within seconds; however, in a study conducted by Malarkey and Parpura [120] exocytosis of most spH-labelled SLMVs upon addition of various stimuli (ATP, bradykinin, Ca^{2+} ionophore 4-Br-A23187, α-latrotoxin, or hypertonicity) occurred with a delay of >1 min and lasted for minutes. Similar to exocytosis of spH-labelled SLMVs, exocytosis of NPY-positive peptidergic vesicles occurred with a delay of more than 1 min upon glutamate [105] or ionomycin stimulation [103]. Moreover, the exocytotic discharge of ANP.emd expressing peptidergic vesicles in 8-Br-cAMP-differentiated astrocytes occurred over a time scale of minutes on ionomycin application [65]. When exocytosis of lysosomes was studied, by labelling astrocytic lysosomes with FM dyes, lysosomes began to exocytose with a delay of more than 1 min on calcium ionophore A-23187 [24], ionomycin, or ATP stimulation [38]. Consistent with this, the exocytotic discharge of the majority of quinacrine-loaded vesicles that express lysosomal TI-VAMP [74] occurs with a delay of more than 2 min with various stimuli including ionomycin, glutamate, ATP or UV-induced Ca^{2+} uncaging stimulation [37, 113]. In addition, EGFP-LAMP1- and FITC-dextran-labelled lysosomes undergo exocytotic discharge with a delay of more than 40 s on either ionomycin stimulation [107] or ATP/DHPG ((R/S)-3,5-dihydroxyphenylglycine) stimulation [106], respectively.

The appearance of antigen-presenting molecules (MHC class II molecules) in endolysosomal compartments, which then get exposed to the cell surface, takes several hours under conditions when astrocytes are stimulated with inflammatory cytokine interferon-γ [39].

These data indicate that compared with neurons, where the fusion occurs within <0.5 ms on Ca^{2+} entry, thereby releasing neurotransmitters into the synaptic cleft [77, 121], the exocytotic release of gliosignal molecules from astrocytes is a much slower process and occurs with a substantial post-stimulus delay. This was most elegantly demonstrated by using C_m measurements on isolated astrocytes, where the kinetics of vesicle fusion in astrocytes [19] was at least two orders of magnitude slower than in neurons, when similar recording techniques were used [122] (Fig. 2). This is more clearly presented in Table 1, where the maximal rate of regulated exocytosis, determined by a similar electrophysiological approach, is compared in different secretory cell types. Astrocytes exhibit a rate even slower than that found in endocrine cells. The relatively slow responsiveness of glial cells makes them an ideal signal integrator suitable for supporting slow signalling mechanisms of the gliocrine

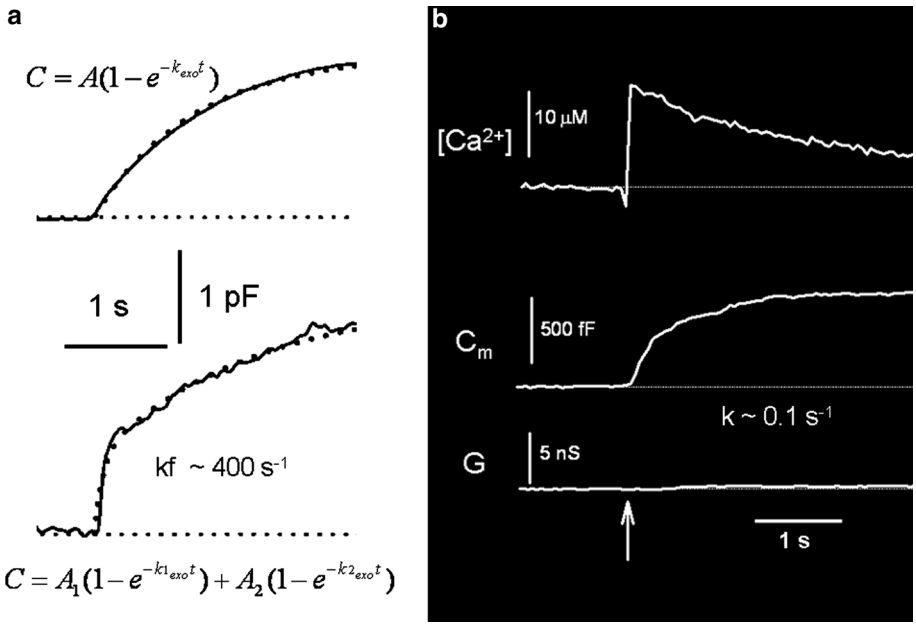

Fig. 2 Comparison of time-dependent changes in C_m recorded in a neuronal cell **a** and in an astrocyte **b**. **a** Two types of Ca^{2+}-induced increases in C_m in a photoreceptor have been recorded. The top trace was best fitted by a single exponential function (*dotted line*), while the *bottom trace* was best fitted to a sum of two exponential functions as shown by the equation below the *horizontal line*. The fastest rate constant (k_f) was around 400 s^{-1}. Modified with permission from Kreft et al. [122]. **b** The *top trace* shows time-dependent changes in $[Ca^{2+}]_i$, elicited by UV light flash photolysis of caged Ca^{2+} compound dialysed into the cytosol of the cell. The UV flash was applied at the time indicated by the *arrow*. Note that the rapid increase in $[Ca^{2+}]_i$ following the UV flash application induced an exponential increase in C_m with a rate constant (k) of 0.1 s^{-1}. G denotes the real part of the admittance trace. Modified with permission from Kreft et al. [19]

Table 1 Comparison of maximal rates of regulated exocytosis in different secretory cell types recorded by flash-photolysis induced cytosolic $[Ca^{2+}]$ increase in whole-cell membrane capacitance records

Cell type	Max rate in regulated exocytosis (s^{-1})	References
Astrocytes	0.1–2	Kreft et al. [19]
Endocrine pituitary cells	25	Thomas et al. [124]
	44	Rupnik et al. [125]
Endocrine pancreatic β cells	70	Barg et al. [126]
		Wan et al. [127]
Rod photoreceptors	300	Thoreson et al. [128]
	400	Kreft et al. [122]
Bipolar neurones	3000	Heidelberger et al. [129]
Calyx of Held neurones	6000	Bollmann et al. [130]
		Schneggenburger and Neher [131]

system, mimicking the function of the endocrine system in the soma. The slowness of the vesicle-mediated signalling may be due to many factors, including different sources of Ca^{2+} between neurones and astrocytes (diffusion of Ca^{2+} from the ER to the plasma membrane), asynchronous coupling between astroglial rapid Ca^{2+} signals and vesicle fusion [24, 113], the slow delivery of vesicles to the plasma membrane fusion sites [123] and/or slow molecular mechanisms governing the merger of the vesicle and the plasma membrane, which needs to be further studied in the future.

Concluding Remarks: A Relatively slow Gliocrine System Maintains Intracranial Homeostasis

The abundance of astrocytes in vivo, at least in the cortex they outnumber neurones by five-fold [8], and their close morphological association with synapses in the CNS (tripartite synapses) and other cells, makes them an ideal partner in CNS intercellular signalling. They are capable of detecting the activity of surrounding cells (neurones, microglia, oligodendrocytes, vascular endothelial cells), and responding back by releasing gliosignals with regulated

vesicle-based mechanisms. This system provides homeostatic control of a number of intracranial physiologic parameters supporting the function of neural networks in the CNS. Detection of the surrounding molecules by the gliocrine system utilizes GPCR-coupled Ca^{2+} signalling mechanisms regulating the excitability of astrocytes. In some cases, such as when NA is released by LC neurons, simultaneous activation of adrenergic GPCRs on astrocytes triggers both intracellular Ca^{2+} and cAMP signalling mechanisms. In contrast to the Ca^{2+}-mediated phasic signalling, cAMP-dependent signalling appears entirely tonic, which makes it most suitable to regulate slow processes. Once astrocytes are excited they use vesicle-based mechanisms to discharge gliosignals, which also affect neighbouring neurones as well as non-neuronal cells at more distant locales from the astrocytic source. This notion is supported by studies of regulated exocytosis in cultured astrocytes, which most likely occurs also in vivo. The essential components of the exocytotic apparatus have been confirmed in astrocytes in situ. Although in vivo the physiologic relevance of regulated vesicular gliosignal release from astrocytes and its importance in normal brain information processing is still under debate, it likely has a role in the modulation of neuronal activity, either in an excitatory or inhibitory manner. Understanding astrocytic vesicular mechanisms in vitro and in vivo under physiologic and pathologic conditions may lead to developments of new targets for therapies.

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