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Mechanisms of glutamate release from astrocytes

Erik B. Malarkey and Vladimir Parpura¹

Departments of Cell Biology & Neuroscience, Centers for Glial-Neuronal Interactions and Nanoscale Science & Engineering, University of California, Riverside, CA 92521

¹ *Departments of Physics & Astronomy, Centers for Glial-Neuronal Interactions and Nanoscale Science & Engineering, University of California, Riverside, CA 92521*

Abstract

Astrocytes can release the excitatory transmitter glutamate which is capable of modulating activity in nearby neurons. This astrocytic glutamate release can occur through six known mechanisms: (i) reversal of uptake by glutamate (ii) anion channel opening induced by cell swelling, (iii) Ca^{2+} -dependent exocytosis, (iv) glutamate exchange via the cystine-glutamate antiporter, (v) release through ionotropic purinergic receptors and (vi) functional unpaired connexons, ‘hemichannels’, on the cell surface. Although these various pathways have been defined, it is not clear how often and to what extent astrocytes employ different mechanisms. It will be necessary to determine whether the same glutamate release mechanisms that operate under physiological conditions operate during pathological conditions or whether there are specific release mechanisms that operate under particular conditions.

Keywords

glutamate release; exocytosis; transporters; P2X receptors; connexin “hemichannels”; anion channels

Introduction

Astrocytes can release a variety of neuroligands into the extracellular space using many different mechanisms. The first demonstration of this astrocytic ability was of primary cultured astrocytes releasing taurine upon β -adrenergic stimulation (Shain and Martin, 1984). In this review we focus on the release of the excitatory neurotransmitter glutamate. This transmitter can be released from astrocytes by several different mechanisms: (i) reversal of uptake by plasma membrane glutamate transporters (Szatkowski et al., 1990), (ii) anion channel opening induced by cell swelling (Kimelberg et al., 1990), (iii) Ca^{2+} -dependent exocytosis (Parpura et al., 1994), (iv) glutamate exchange via the cystine-glutamate antiporter (Warr et al., 1999), (v) release through ionotropic purinergic receptors (Duan et al., 2003) and (vi) functional unpaired connexons, ‘hemichannels’, on the cell surface (Ye et al., 2003). We discuss studies using variety of different approaches which provided evidence for the different mechanisms of glutamate release in astrocytes. While Ca^{2+} -dependent vesicular release of glutamate from astrocytes can readily occur under physiological conditions, there is some question as to

Correspondence: Vladimir Parpura, Department of Cell Biology & Neuroscience, Center for Glial-Neuronal Interactions, 1208 Spieth Hall, University of California, Riverside, CA 92521, Phone/Fax: (951) 827-2074, E-mail: vlad@ucr.edu.

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whether some mechanisms might solely operate during pathophysiological circumstances, such as ischemia or stroke, since they may require conditions like cell swelling or a low extracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_o$) to occur. An understanding of these mechanisms and conditions that underlie glutamate release will provide information on glial functions in health and disease and may also introduce opportunities for medical intervention.

Ca^{2+} - dependent exocytosis

The first evidence for Ca^{2+} - dependent release of glutamate from cultured astrocytes was shown in experiments using high performance liquid chromatography, where bradykinin-evoked intracellular Ca^{2+} elevations in cultured astrocytes resulted in the release of glutamate. This release could also be visualized by N-methyl-D-aspartic acid (NMDA) receptor-mediated intracellular Ca^{2+} increases in surrounding neurons (Parpura et al., 1994). An increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) is sufficient and necessary to cause glutamate release from astrocytes. Hence, when the Ca^{2+} ionophore, ionomycin, was applied to astrocytes it stimulated the release of glutamate in the presence of external free Ca^{2+} (2.4 mM). However, ionomycin failed to cause glutamate release when internal Ca^{2+} stores were depleted by preventing Ca^{2+} entry from the extracellular space by bathing the astrocytes in a solution of low external free Ca^{2+} (24 nM) for 40–60 minutes (Parpura et al., 1994). Further studies supported this conclusion since buffering cytoplasmic Ca^{2+} with 1,2-bis(o-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA) or depleting internal calcium stores by application of thapsigargin, a blocker of store specific Ca^{2+} -ATPase, also resulted in a reduction of glutamate release (Araque et al., 1998b; Bezzi et al., 1998). Using flash photolysis of a caged Ca^{2+} compound to evoke graded rises of cytoplasmic Ca^{2+} concentration in astrocytes, demonstrated that glutamate release can result from moderate increases in Ca^{2+} concentration that are likely to occur physiologically (Parpura and Haydon, 2000). Indeed, supporting evidence for Ca^{2+} -dependent glutamate release was, subsequently, established in acute slice preparations (Bezzi et al., 1998). Additionally, this release mechanism was determined to be distinct from swelling or reverse operation of plasma membrane glutamate transporters since no change in astrocyte volume was detected and glutamate transporter inhibitors did not abolish the release (Parpura et al., 1995b; Jęftinija et al., 1996; Araque et al., 2000; Innocenti et al., 2000).

The Ca^{2+} necessary for this glutamate release comes from two sources. The majority originates from internal stores, but entry of external Ca^{2+} is also involved. This was demonstrated by the reduction of mechanically-induced glutamate release in the presence of thapsigargin and also by Cd^{2+} , a blocker of Ca^{2+} entry from the extracellular space (Hua et al., 2004). This release requires co-activation of inositol 1,4,5-trisphosphate (IP_3)- and ryanodine/cafeine-sensitive internal Ca^{2+} stores, which operate jointly (Hua et al., 2004). Currently, the identity of the mechanism mediating Ca^{2+} entry from the extracellular space, involved with mechanically-induced glutamate release in astrocytes is unresolved. Transient receptor potential (TRP) proteins, which are believed to be activated by depletion of internal Ca^{2+} stores to allow Ca^{2+} entry from the extracellular space, have been implicated in the regulation of astrocytic Ca^{2+} homeostasis (Pizzo et al., 2001; Grimaldi et al., 2003; Golovina, 2005; Malarkey and Parpura, 2005); naturally, these proteins are the prime candidates for mediating the entry of external Ca^{2+} in mechanically-induced glutamate release from astrocytes.

The Ca^{2+} -dependency of glutamate release from astrocytes suggests regulated exocytosis as a possible mechanism underlying this release. Astrocytes possess the secretory machinery for regulated exocytosis which utilizes a complex of proteins, the soluble N-ethyl maleimide-sensitive fusion protein attachment protein receptor (SNARE) complex, to control vesicle fusion [for review see (Montana et al., 2004)]. This includes proteins of the core SNARE complex: synaptobrevin 2, Syntaxin 1 and synaptosome-associated protein of 23 kDa

(SNAP-23) (Parpura et al., 1995a; Jeftinija et al., 1997; Hepp et al., 1999; Maienschein et al., 1999; Araque et al., 2000; Pasti et al., 2001; Montana et al., 2004; Crippa et al., 2006) and a Ca^{2+} sensor Synaptotagmin 4 (Zhang et al., 2004a; Crippa et al., 2006). Proteins important for sequestering of glutamate into vesicles have also been discovered in astrocytes. Vacuolar type H^{+} -ATPase (V-ATPase), which creates the proton concentration gradient necessary for glutamate transport into vesicles (Araque et al., 2000; Bezzi et al., 2001; Pasti et al., 2001; Montana et al., 2004; Wilhelm et al., 2004); (Crippa et al., 2006) and the three known isoforms of vesicular glutamate transporters (VGLUT): 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), have been detected in astrocytes and play a functional role in mediating Ca^{2+} -dependent glutamate release from astrocytes (Figure 1A, Table 1). The action of specific pharmacological agents and molecular biological manipulations that affect glutamatergic exocytosis was confirmed in astrocytes [reviewed in (Montana et al., 2004)]

The presence of vesicular proteins in astrocytes, including VGLUTs, implies that astrocytes release glutamate via a vesicular pathway. Such a notion requires evidence of the existence of astrocytic secretory vesicles, since these organelles are the essential morphological elements for regulated, Ca^{2+} -dependent exocytosis. Secretory granules in the glia of grey matter were described nearly 100 years ago (Nageotte, 1910). However, only recently, has there been compelling evidence for morphological correlates of the exocytotic process underlying glutamate release in astrocytes [reviewed in (Montana et al., 2004)]. An immunoelectron microscopy (IEM) study demonstrated that synaptobrevin 2 could be associated with electron-lucent (clear) vesicular structures with diameters ranging from 100–700 nm (Maienschein et al., 1999). The IEM of VGLUTs 1 or 2 in astrocytes *in situ* showed an association of these proteins with small clear vesicles with a mean diameter of ~30 nm (Bezzi et al., 2004). Additionally, VGLUT 2 was found on synaptobrevin 2-containing vesicles immunisolated from cultured astrocytes (Crippa et al., 2006). These vesicles were shown to be predominantly clear and heterogeneous in size, ranging from 30 to over 100 nm. Furthermore, the presence of clear smooth and clathrin-coated vesicles with apparent diameters of ~30 nm has been observed in gliosomes, a purified preparation of re-sealed fragments of astrocytes from the adult rat brain (Stigliani et al., 2006), which expressed synaptobrevin 2 and VGLUT 1.

The delivery of small synaptic-like vesicles to plasma membrane exocytotic sites has been investigated in astrocytes. Crippa et al. (2006) expressed chimeric protein where enhanced green fluorescent protein (EGFP) was fused to the C-terminus of synaptobrevin 2 (synaptobrevin 2-EGFP) in astrocytes. Synaptobrevin 2-EGFP, disclosing the location of small vesicles, showed a punctate pattern of fluorescence throughout the cells. These astrocytic vesicles displayed mobility behavior similar to that of synaptic vesicles in neurons. When astrocytes were stimulated to exhibit regulated exocytosis, many fluorescent synaptobrevin 2-EGFP puncta disappeared with a concomitant increase in plasma membrane fluorescence, consistent with full fusion of labeled vesicles. During the process of exocytosis, there is a net addition of vesicular membrane to the plasma membrane, which can be directly assessed by monitoring changes in plasma membrane capacitance (C_m). Consequently, stimulation to increase $[\text{Ca}^{2+}]_i$ in astrocyte using t-ACPD caused an increase C_m , while simultaneous measurements recorded release of glutamate (Zhang et al., 2004b). Further evidence for vesicular exocytosis from astrocytes was provided by Bezzi et al. (2004). Using total internal reflection fluorescence microscopy, they followed up on the spatio-temporal characteristics of exocytosis of VGLUT positive vesicles. Similarly, glutamatergic exocytosis in astrocytes has been demonstrated by amperometric measurements used to detect the release of dopamine, acting as a “surrogate” transmitter for glutamate, from glutamatergic vesicles (Chen et al., 2005). Finally, the process of exocytosis is characterized by quantal release of neurotransmitter

(Del Castillo and Katz, 1954) and quantal-like events of glutamate release have been recorded from astrocytes (Pasti et al., 2001)

Future experiments will be needed to define the location of the exocytotic release sites *in vivo* and the contribution of this release from astrocytes to the physiology and pathophysiology of the central nervous system. One of the emerging functions in which this release may play a role is the spatial coordination and synchronization of neuronal activity and synaptic networks (Angulo et al., 2004; Fellin et al., 2004; Pascual et al., 2005).

Reverse operation of glutamate transporters

An important function that astrocytes perform is the removal of glutamate from extracellular space. This function is accomplished through the use of plasma membrane Na^+ -dependent amino acid transporters which couple the transport of Na^+ and K^+ down their respective concentration gradients to driving glutamate into the cell (Anderson and Swanson, 2000). The majority of glutamate transporters found in astrocytes are associated with synapses (Chaudhry et al., 1995; Minelli et al., 2001). Hence, the removal of glutamate from the extracellular space by astrocytes aids in termination of synaptic neurotransmission and prevents excitotoxicity (Rothstein et al., 1996; Bergles and Jahr, 1997).

Astrocytes predominantly express two transporters that are used in this process: the L-glutamate/L-aspartate transporter (GLAST-1) and the glial L-glutamate transporter (GLT-1), also called excitatory amino acid transporters (EAAT1 and EAAT2, respectively) (Gadea and Lopez-Colome, 2001). Normally, concentration gradients favor the transport of glutamate into astrocytes. However, during pathophysiological events, such as ischemia, perturbed ionic conditions (e.g. increased extracellular K^+ levels) may favor transporters operating in reverse (Figure 1B). Transport reversal was first demonstrated in glial cells by measuring glutamate induced currents while raising extracellular K^+ levels (Szatkowski et al., 1990). Although it has been shown that under normal physiological conditions, extracellular K^+ levels could not be elevated enough to cause reverse transport of glutamate out of cultured astrocytes (Longuemare and Swanson, 1997), there have been numerous cases, using glutamate transporter inhibitors, showing that reverse transport of glutamate can occur during periods of ischemia or metabolic blockade (Longuemare and Swanson, 1995; Zeevalk et al., 1998; Li et al., 1999; Seki et al., 1999; Rossi et al., 2000) (Table 1). Accordingly, by using the EAAT substrate inhibitor L-trans-pyrrolidine-2,4-dicarboxylate (PDC) to induce glutamate release by heteroexchange (Volterra et al., 1996), reverse transport was shown to be a major event leading to cell death in astrocytes (Re et al., 2006). However, cell death was not caused by the increase in extracellular glutamate, but by the depletion of cytoplasmic glutathione resulting from the loss of glutamate inside the cell, which then leads to oxidative death.

Cystine-glutamate antiporter

Cystine uptake in cells is important for the production of the antioxidant, glutathione. Uptake can occur through either the plasma membrane Na^+ -independent cystine-glutamate exchanger (system x_c^-) or the Na^+ -dependent glutamate transporters (system X_{AG}^-) [reviewed in (McBean, 2002)]. Although results differ in the amount of uptake contributed by each process, astrocytes have been shown to utilize both of them in cystine uptake (Bender et al., 2000; Allen et al., 2001; Shanker et al., 2001). The majority of cystine-glutamate exchanger localization in the brain occurs in glial cells (Pow, 2001) as revealed by immunolocalization of aminoadipic acid, a selective substrate for this exchanger.

Since the x_c^- system functions by importing cystine in exchange for glutamate, this may provide a pathway for glutamate release from astrocytes (Figure 1C, Table 1) This has been initially demonstrated in cerebellar slices, where addition of cystine to the extracellular space generated

currents in Purkinje cells. These currents were attributed to glutamate released from glia, since the currents were abolished by glutamate receptor antagonists and were not seen in isolated Purkinje cells (Warr et al., 1999). Applying homocysteic acid or (S)-4-carboxyphenylglycine (CPG) to block the cystine-glutamate exchanger decreased the extracellular glutamate concentration in rat striatum by 60% (Baker et al., 2002). Agonists or antagonists of mGluR2 receptors were shown to down- or up-regulate, respectively, glutamate release by the cystine-glutamate exchanger (Tang and Kalivas, 2003) and the glutamate released through the cystine-glutamate exchanger has also been shown to reduce both spontaneous and action-potential evoked glutamate release, again, by action on mGluR2, although in a brain region dependent manner (Moran et al., 2003).

Cavelier and Atwell (2005) have raised questions of whether tonic release of glutamate through the x_c^- system occurs under normal physiological conditions. They found that blocking the cystine-glutamate exchanger had no effect on glutamate release unless unphysiologically high levels of external cystine were present. They also pointed out that physiological levels of intercellular cystine found in the brain were well below the reported EC_{50} of the exchanger [(Warr et al., 1999), but see (Baker et al., 2003)]. However, the role of glutamate release by the cystine-glutamate exchanger *in vivo* has been demonstrated by Moran et al. (2005). Here first using acute slices the authors found that application of physiological levels of cystine (100–300 nM) could elevate extracellular glutamate concentrations and reduce excitatory synaptic activity; this effect was blocked by application of CPG or the mGluR2/3 antagonist LY341495. Since a reduction in extracellular glutamate by withdrawal from cocaine is attributed to compromised x_c^- system function (Baker et al., 2003), behavioral studies with rats were performed (Moran et al., 2005). Restoring extracellular glutamate with systemic administration of cysteine prodrugs prevented the reinstatement of cocaine seeking and this effect was reversed by the application of mGluR2/3 antagonist, indicating that glutamate released via the x_c^- system stimulates inhibitory presynaptic mGluR2/3 receptors which reduce synaptic glutamate release, preventing drug seeking. Conversely, inhibiting the function of the cystine-glutamate exchanger with CPG or by removing extracellular cystine, has been shown to cause cell death in astrocytes, presumably by oxidative death due to lack of cystine to convert to glutathione (Re et al., 2006). A similar approach was employed for possible clinical benefit by using sulfasalazine to block system x_c^- in gliomas. This treatment reduced the levels of intracellular glutathione and consequently caused oxidative cell death that resulted in a reduction in growth of gliomas (Chung et al., 2005).

P2X₇ receptors

The purinergic P2X₇ ion channel may provide another pathway for glutamate release from astrocytes. P2X receptors are adenosine 5'-triphosphate (ATP)-gated, cation selective, ion channels that show amplified responses in low external divalent cation solution. There are seven known types of P2X receptor subunits that can assemble to form homomeric or heteromeric channels. The homomeric P2X₇ receptor possesses a pore that is able to allow molecules as large as 900 Da to pass through (North, 2002). The P2X₇ receptor has been detected in astrocytes *in vitro* by RT-PCR (Fumagalli et al., 2003), immunoblotting and immunolocalization (Duan et al., 2003), and there is indication that they may be up-regulated after injury (Franke et al., 2001). P2X₇ receptors have also been detected in astrocytes *in vivo* using hippocampal sections of juvenile rats (Kukley et al., 2001). Astrocytic localization was determined by immunocytochemistry using antibodies against P2X₇, along with S100 β as an astrocytic marker.

Duan et al. (2003) provided the first evidence that these channels could mediate the release of glutamate from astrocytes. Application of ATP to cultured astrocytes expressing P2X₇ receptors induced an inward current that was augmented by low divalent cation external

solution. Activation of these receptors enabled astrocytic uptake of the fluorescent dye, Lucifer Yellow (LY), which was also increased in low divalent cation external solution. Current or dye uptake was inhibited by the P2 receptor antagonist, pyridoxal phosphate-6-azophenyl-2-4-disulfonic acid (PPADS), the anion channel blocker, DIDS, or the more specific P2X₇ antagonist, oxidized ATP (oATP). The induced current was also insensitive to voltage changes, which differentiates it from channels such as gap junctions. Release of radiolabeled glutamate was induced by application of ATP, but more potently with 3'-O-(4-benzoyl)benzoyl ATP (BzATP), and increased in low divalent cation solution, consistent with release through the P2X₇ channel, while PPADS, DIDS or oATP blocked its release. This glutamate release does not seem to occur by a mechanism that requires $[Ca^{2+}]_i$ increase since preincubating the cells with the membrane permeable calcium chelator, acetoxymethyl ester of BAPTA, did not reduce the amount of released glutamate.

In hippocampal slices, Fellin et al. (2006) found that perfusion of BzATP induced transient slow inward currents (SICs) and tonic currents in pyramidal neurons that resulted from NMDA receptor activation. The currents were induced by glutamate release from astrocytes, since they occurred in the presence of tetrodotoxin. The tonic current, but not SICs, appeared to be mediated by glutamate release from P2X₇-like receptors as it was blocked by the P2X antagonists, oATP and Brilliant Blue G (BBG) and were enhanced in low Ca^{2+} external solution. Glutamate release through transporter reversal or hemichannels was ruled out since the glutamate transporter inhibitor, DL-threo- β -benzyloxyaspartate (TBOA), or carboxoxolone did not affect the tonic current. In astrocytes BzATP induced an inward current that was blocked by BBG and not affected by any glutamate receptor antagonists, so the current did not result from released glutamate. BzATP was also able to induce uptake of LY in astrocytes, which was again blocked by BBG. This work indicates that ATP *in situ* can cause release of glutamate from astrocytes through P2X receptors that provide tonic stimulation of surrounding neurons.

Swelling/anion channels

Under hypo-osmotic conditions, such as those occurring during ischemia, most cells experience swelling and can compensate for this volume increase by opening volume-regulated anion channels (VRACs). These channels are permeable to inorganic and small organic anions, including the amino acids taurine, aspartate and glutamate (Mongin and Orlov, 2001). Release of glutamate from cultured astrocytes during hypo-osmotically induced swelling was first reported by Kimelberg et al. (1990) using radiolabeled glutamate. They found that this release of glutamate occurred through an anion channel since it could be blocked by various anion channel inhibitors (Figure 1E, Table 1). Another study showed that glutamate released from astrocytes contributed to spreading depression in hippocampal slices. This glutamate was released in Ca^{2+} free medium but blocked by 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), a Cl^- channel inhibitor, indicating that glutamate could have been released through VRACs (Basarsky et al., 1999). Liu et al. (2006) discerned swelling induced glutamate release from two different anion channels, the volume-sensitive outwardly rectifying (VSOR) chloride channels and the maxi-anion channels. Using hyposmotic solution or inducing metabolic arrest resulted in release of glutamate from astrocytes that was not mediated by hemichannels or vesicular release. Using cell attached patches they found channels with large conductance that were insensitive to phloretin but inhibited by Gd^{3+} , representative of maxi-anion channels. The swelling- and ischemia-induced release of glutamate was greatly suppressed by blockers of maxi-anion channels such as NPPB, 4-acetamido-4'-isothiocyanato-stilbene-2,2'-disulfonate (SITS), Gd^{3+} , and arachidonic acid. This result strongly suggests that the maxi-anion channel is the major contributor to the release of glutamate from cultured astrocytes. However, not all glutamate release was prevented by blockers of maxi-anion channels and whole cell and cell

attached patch currents indicative of VSOR were detected which were blockable by phloretin and tamoxifen.

There have been many reports that astrocytes can release amino acids during pathologically induced swelling [reviewed in (Kimelberg, 2005)], but non-pathological glutamate release through VRACs has not been demonstrated. The small amount of volume change that may occur in astrocytes under physiological conditions seems insufficient to cause significant glutamate release. However, it has been shown that compounds such as ATP (Mongin and Kimelberg, 2002, 2005; Takano et al., 2005) or nitric oxide (Ellershaw et al., 2000) can act on VRACs to amplify amino acid release under conditions of only moderate swelling or hypo-osmolarity which could possibly occur *in vivo*. There is evidence that receptor mediated intracellular Ca^{2+} increases, induced by ATP in astrocytes, can result in transient cell swelling leading to glutamate release through VRACs (Takano et al., 2005). Using whole-cell recoding, application of ATP caused the opening of channels which, along with glutamate release, was reduced by BAPTA and the anion channel blockers: NPPB, flufenamic acid and gossypol, but neither a glutamate transporter inhibitor nor two compounds known to affect vesicular release, tetanus toxin and bafilomycin A1. Glutamate release did not appear to be through P2X_7 channels since BzATP had no effect and the channels were not cation selective. Also connexin hemichannels, most likely, did not play a role since ATP-induced glutamate release was similar in astrocytes from Cx43 knockout and wild type mice.

Determining conclusively that glutamate is released through VRACs has proven complicated due to a lack of specific inhibitors of suspected pathways. For example, many of the anion channel inhibitors used have been shown to block connexin hemichannels (Eskandari et al., 2002). Further, the anion channel blockers used in most studies of VRACs have been reported to act on a variety of other channels. For example, NPPB can inhibit the vesicular chloride transporter and 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate (DIDS) has been implicated in blocking VGLUTs and V-ATPases (reviewed in Evanko et al., 2004)]. Although buffering cytoplasmic Ca^{2+} can interfere with osmotically-induced amino acid release from astrocytes (Mongin et al., 1999), tetanus toxin had no effect on swelling-induced release, indicating that this release does not appear to be through a vesicle mediated pathway (Mongin and Kimelberg, 2002). This also emphasizes how difficult it can be to isolate the effects of one release mechanism from another.

Connexons/Pannexons

Gap junction channels form a pore between two adjacent cells, connecting their cytoplasm, and allowing molecules as large as about 1 kDa to diffuse between cells. These gap junctions are formed by the joining of two connexons ("hemichannels") each composed of a hexamer of the protein connexin. Although there are many different isoforms of connexin, Cx43 appears to be the most prevalent in astrocytes (Dermietzel et al., 2000). There is evidence that unpaired connexons may be able to act as functional hemichannels, capable of opening to the external space (Hofer and Dermietzel, 1998; Contreras et al., 2002; Stout et al., 2002; Ye et al., 2003).

Since these hemichannels can pass large molecules, then their opening could provide a mechanism whereby transmitters such as glutamate could diffuse out of astrocytes (Figure 1F, Table 1). There has been some evidence supporting this kind of release through hemichannels for glutamate release (Ye et al., 2003). Ye et al. (2003) found that under conditions of low extracellular divalent cations, hippocampal astrocytes showed release of glutamate. This release, and also LY dye uptake, was reduced by application of the gap junction blockers, carbenoxolone, heptanol, octanol and 18α -glycyrrhetic acid, as well as by multivalent cations. However, release was not inhibited by the P2X receptor blockers, oATP and PPADS or the chloride channel blocker, DIDS. This seems consistent with release through hemichannels, but, as stated above, many compounds used as gap junction blockers have been

shown to affect anion channels as well (Eskandari et al., 2002). Several channels could provide a means for the glutamate release from astrocytes attributed to connexin hemichannels: pannexins, P2X receptors and also various anion channels such as, voltage dependent anion conductance (VDAC), cystic fibrosis transmembrane conductance regulator (CTFR), or VSOR have all been shown to operate under similar conditions and respond to chemical agents commonly used to manipulate hemichannels. Often, the involvement of hemichannels in mediating a given event is claimed using insufficient criteria, such as reduction of glutamate release using a gap junction blocker that is non-specific. A set of stringent criteria have been proposed in order to establish whether an observed effect should be attributed to hemichannels (for a discussion of this issue see: Spray et al., 2006). However, astrocytes cultured from Cx43 knock-out mice and exposed to low extracellular divalent cations show minimal LY dye uptake and glutamate release, when compared to astrocytes originating from control wild type animals (Spray et al., 2006). Such finding supports the notion that glutamate could, indeed, be released via connexin hemichannels. This may appear at odds with the voltage sensitivity of gap junction channels, which only open as membrane potentials become positive (Trexler et al., 1996), an event that normally does not occur with astrocytes. However, there is some evidence that hemichannels can be gated under resting conditions (Contreras et al., 2003; Saez et al., 2005). Whether release through hemichannels would occur during normal conditions in the brain, or perhaps only during injury or ischemia, still needs to be determined.

The pannexin family of proteins can also form conductive channels similar to the connexins. Non-junctional pannexin channels, “pannexons”, are not sensitive to extracellular Ca^{2+} and can be opened by cytoplasmic Ca^{2+} elevations at membrane potentials within the range normally observed in astrocytes (Bruzzone et al., 2003). Pannexins are also sensitive to several of the same compounds used to block connexon hemichannels, such as carbenoxolone and flufenamic acid (Bruzzone et al., 2005). Many of the properties attributed to connexon hemichannels correlate well with those of pannexons, and with the detection of pannexin-1 (Pelegrin and Surprenant, 2006; Lai et al., 2007) 2 and 3 (Lai et al., 2007) RNA in astrocytes, pannexon hemichannels may have a role in mediating glutamate release from astrocytes.

Concluding remarks

The purpose of this review was to summarize the underlying mechanisms of glutamate release from astrocytes. There are several different mechanisms that can mediate glutamate release from these cells, as outlined in Figure 1, however, there are issues that remain to be resolved. It will be necessary to determine whether the same glutamate release mechanisms that operate under physiological conditions operate during pathological conditions or whether there are specific release mechanisms that operate under particular conditions. Alternatively, all possible mechanisms of glutamate release could operate together in astrocytes at all times, but each contributing a different portion to the total amount of glutamate being released. The major impediment in systematically conducting studies to address these issues is the absence of specific reagents and methods for teasing apart the contributions from individual release pathways, as was alluded to earlier. So far the most developed tools relate to the mechanism of exocytosis, this is owing to the over 50 years of work by many laboratories studying the exocytotic process in neurons. However, the development of novel genetic approaches that, for example, selectively manipulate exocytotic release from astrocytes (Pascual et al., 2005) or the use of Cx43 knock out animals provide good starting points to rigorously test the contribution of different glutamate release pathways in astrocytes in health and disease.

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Figure 1. Mechanisms of glutamate release from astrocytes

A) Ca^{2+} -dependent exocytosis, **B)** reversal of uptake by plasma membrane glutamate transporters, **C)** glutamate exchange via the cystine-glutamate antiporter, **D)** release through ionotropic purinergic receptors (only one subunit shown, but exchanger is believed to be multimeric), **E)** anion channel opening induced by cell swelling, **F)** release through functional unpaired connexons, 'hemichannels'. Abbreviations: Sb 2, synaptobrevin 2; SNAP-23, synaptosome-associated protein of 23 kDa; Stg 4, synaptotagmin 4; Sx 1, syntaxin 1; V-ATPase, Vacuolar type H^+ -ATPase; VGLUT, vesicular glutamate transporters.

Table 1

Mechanisms of glutamate release from astrocytes and compounds shown to affect them

Mechanism	Stimulator	[Ca ²⁺] _i Dep.	Inhibitor	References
Vesicular	2MeSADP	Yes	BAPTA	(Domercq et al., 2006)
			A3P5PS	(Domercq et al., 2006)
			Aspirin	(Domercq et al., 2006)
			Bafilomycin A1	(Domercq et al., 2006)
			BB 3103	(Domercq et al., 2006)
			Cyclopiazonic acid	(Domercq et al., 2006)
			Indomethacin	(Domercq et al., 2006)
			PD98059	(Domercq et al., 2006)
			sTNFR1	(Domercq et al., 2006)
			Tetanus Toxin	(Domercq et al., 2006)
			TNF α Antibody	(Domercq et al., 2006)
	4-Br A23187 α -Latrotoxin	Yes Yes/ No		(Innocenti et al., 2000)
				(Parpura et al., 1995b; Jęftinija et al., 1996; Crippa et al., 2006; Bezzi et al., 2001)
				(Fellin et al., 2004; Fellin et al., 2006)
	$\alpha\beta$ mATP t-ACPD t-ACPD+AMPA	Yes		(Bezzi et al., 1998; Zhang et al., 2004b)
			0 Na ⁺ External	(Bezzi et al., 1998)
			Aristolochic acid	(Bezzi et al., 1998)
	ATP	Yes	Aspirin	(Bezzi et al., 1998)
			BAPTA	(Bezzi et al., 1998)
			CNQX	(Bezzi et al., 1998)
			Cyclopiazonic acid	(Pasti et al., 2001)
			EGTA	(Bezzi et al., 1998)
			Furosemide	(Bezzi et al., 1998)
			GYKI 52466	(Bezzi et al., 1998)
			Indomethacin	(Bezzi 1998, Pasti et al., 2001)
			MCPG	(Bezzi et al., 1998)
			pBPB	(Bezzi et al., 1998)
			Tetanus Toxin	(Bezzi 1998, Pasti et al., 2001)
			BAPTA	(Zhang et al., 2004b; Jeremic et al., 2001); Domercq et al., 2006)
			2-APB	(Zhang et al., 2004b)
			A3P5PS	(Domercq et al., 2006)
			Bafilomycin A1	(Zhang et al., 2004b)
			Furosemide	(Jeremic et al., 2001)
			NPPB	(Jeremic et al., 2001)
			PPADS	(Jeremic et al., 2001; Domercq et al., 2006)
			Rose Bengal	(Montana et al., 2004)
			SNARE-domain	(Zhang et al., 2004b)
			Suramin	(Jeremic et al., 2001; Domercq et al., 2006)
			Stg 4 siRNA	(Zhang et al., 2004a)
			Stg 4 dominant negative mutation	(Zhang et al., 2004a)
	BDNF	Yes	Thapsigargin	(Jeremic et al., 2001)
			U73122	(Jeremic et al., 2001)
			BAPTA	(Pascual et al., 2001)
	Bradykinin	Yes	EGTA	(Pascual et al., 2001)
			K252a	(Pascual et al., 2001)
			Tetanus Toxin	(Pascual et al., 2001)
			BAPTA	(Jęftinija et al., 1996)
			Botulinum Toxin A	(Jęftinija et al., 1997)
			Botulinum Toxin B	(Jęftinija et al., 1997)
			Botulinum Toxin C	(Jęftinija et al., 1997)
			EGTA	(Jęftinija et al., 1996)
			Furosemide	(Parpura et al., 1994)
			Rose Bengal	(Montana et al., 2004)
			Tetanus Toxin	(Jęftinija et al., 1997)
	BzATP CHPG DHPG	Yes Yes Yes		(Bezzi et al., 1998)
				(Fellin et al., 2006)
				(Fellin et al., 2004)
	Glutamate H ₂ O ₂ HIV-1 gp120 IIIB	Yes Yes Yes	BAPTA	(Bezzi et al., 2004)
			Tetanus Toxin	(Bezzi et al., 2004)
				(Fellin et al., 2004)
				(Chen et al., 2005)
			BAPTA/EGTA	(Gonzalez et al., 2006)
			AMD 3100	(Bezzi et al., 2001)
			Aspirin	(Bezzi et al., 2001)
			Bafilomycin A1	(Bezzi et al., 2001)
			BB 3103	(Bezzi et al., 2001)
			Indomethacin	(Bezzi et al., 2001)
				(Bezzi et al., 2001)

Mechanism	Stimulator	[Ca ²⁺] _i Dep.	Inhibitor	References
	Ionomycin	Yes	mAb 12G5 sTNFR1 Tetanus Toxin EGTA	(Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Parpura et al., 1994; Jęftinija et al., 1996; Chen et al., 2005)
	IP ₃	Yes	Tetanus Toxin	(Hua et al., 2004; Crippa et al., 2006)
	Low Ca ²⁺	Yes		(Takano et al., 2005)
	Mechanical	Yes	2-APB Bafilomycin A1 BAPTA	(Fellin et al., 2004) (Hua et al., 2004) (Araque et al., 2000; Montana et al., 2004; Hua et al., 2004) ((Araque et al., 1998a)Araque et at., 1998a; Innocenti et al., 2000; Montana et al., 2004; Hua et al., 2004)
			Botulinum Toxin B Caffeine Cd ²⁺ EGTA Rose Bengal Ryanodine SNARE-motif Tetanus Toxin Thapsigargin	(Araque et al., 2000) (Hua et al., 2004) (Hua et al., 2004) (Chen et al., 2005) (Montana et al., 2004) (Hua et al., 2004) (Zhang et al., 2004b) (Montana et al., 2004; Hua et al., 2004; Chen et al., 2005) (Araque et al., 1998b; Innocenti et al., 2000; Hua et al., 2004)
	Nitric Oxide	Yes	BAPTA Botulinum Toxin C EGTA Gd ³⁺ PTIO	(Bal-Price et al., 2002) (Bal-Price et al., 2002) (Bal-Price et al., 2002) (Bal-Price et al., 2002) (Bal-Price et al., 2002)
	NP-EGTA	Yes		(Araque et al., 1998a; Parpura and Haydon, 2000; Fellin et al., 2004)
	Prostaglandin E ₂	Yes	BAPTA	(Bezzi et al., 1998; (Sanzgiri et al., 1999); Bezzi et al., 2001)
			Bafilomycin A1 Tetanus Toxin Thapsigargin	(Bezzi et al., 2001) (Bezzi et al., 2001) (Sanzgiri et al., 1999)
	Photostimulation	Yes	EGTA	(Chen et al., 2005)
	L-quisqualate	Yes	BAPTA Bafilomycin A1 Tetanus Toxin	(Parpura et al., 1994) (Crippa et al., 2006) (Pasti et al., 2001)
	SDF-1α	Yes	BAPTA AMD 3100 Aspirin Bafilomycin A1 BB 3103 Captopril Indomethacin mAb 12G5 PD98059 sTNFR1 Tetanus Toxin TNFα Antibody UO126 Aspirin Bafilomycin A1 BAPTA Indomethacin	(Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Bezzi et al., 2001) (Rossi et al., 2005) (Rossi et al., 2005) (Bezzi et al., 2001; Rossi et al., 2005)
P2X ₇ Receptor	ATP	No	PPADS	(Duan et al., 2003)
	BzATP	No	DIDS OxATP PPADS BBG DIDS OxATP	(Duan et al., 2003) (Duan et al., 2003) (Duan et al., 2003) (Fellin et al., 2006) (Duan et al., 2003) (Duan et al., 2003; Fellin et al., 2006)
Hemichanne l	Divalent Cation (div cat.) free solution	No*	18α-glycyrrhetic acid Ca ²⁺ (other div. cat.) Cx43 KO	(Ye et al., 2003) (Ye et al., 2003; Spray et al., 2006) (Spray et al., 2006)

Mechanism	Stimulator	[Ca ²⁺] _i Dep.	Inhibitor	References
			Flufenamic acid Heptanol Hexanol Octanol	(Ye et al., 2003) (Ye et al., 2003) (Ye et al., 2003) (Ye et al., 2003)
Anion Channel	Hypotonic Solution	No	Arachadonic Acid	(Liu et al., 2006)
			Flufenamic acid Furosemide Gd ³⁺ Gossypol L-644,711 NPPB Phloretin SITS Tamoxifen	(Takano et al., 2005) (Kimelberg et al., 1990) (Liu et al., 2006) (Takano et al., 2005) (Kimelberg, et al., 1990) (Takano et al., 2005; Liu et al., 2006) (Liu et al., 2006) (Kimelberg et al., 1990; Liu et al., 2006) (Liu et al., 2006) (Jeftinija et al., 1997; Zhang et al., 2004*)
	Ouabain 2DG/cyanide/IA	No No	NPPB NPPB Arachadonic Acid Gd ³⁺ Phloretin SITS Tamoxifen	(Basarsky et al., 1999) (Liu et al., 2006) (Liu et al., 2006) (Liu et al., 2006) (Liu et al., 2006) (Liu et al., 2006) (Liu et al., 2006)
	ATP	Yes	BAPTA Flufenamic acid Gossypol Hyperosmolarity NPPB Reactive Blue 2 Thapsigargin	(Takano et al., 2005) (Takano et al., 2005) (Takano et al., 2005) (Takano et al., 2005) (Takano et al., 2005) (Takano et al., 2005) (Takano et al., 2005)
Reverse Transport	High K ⁺ external	No	D-Aspartate Glutamate Na ⁺	(Szatkowski et al., 1990) (Szatkowski et al., 1990) (Szatkowski et al., 1990; (Longuemare et al., 1999) (Longuemare et al., 1999) (Longuemare et al., 1999) (Zeevalk et al., 1998) (Bezzi et al., 1998; Re et al., 2006) (Kimelberg et al., 1990; Re et al., 2006) (Re et al., 2006)
	Fluoride/Azide IA/Cyanide PDC TBHA TBOA	No No	SITS PDC PDC	(Longuemare et al., 1999) (Longuemare et al., 1999) (Longuemare et al., 1999) (Zeevalk et al., 1998) (Bezzi et al., 1998; Re et al., 2006) (Kimelberg et al., 1990; Re et al., 2006) (Re et al., 2006)
Cystine-Glutamate Antiporter	Cystine	No	CPG CPG Homocysteic acid	(Moran et al., 2003) (Warr et al., 1999) (Baker et al., 2002) (Baker et al., 2002)

Abbreviations. 2-APB, 2-aminoethoxydiphenyl borate; 2DG, 2-Deoxy-D-glucose; 2MeSADP, 2-methylthio-adenosine diphosphate; $\alpha\beta$ mATP, α,β -methylene ATP; A3P5PS, adenosine 3'-phosphate, 5'-phosphosulfate; AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ATP, adenosine 5'-triphosphate; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; BB 3103, a blocker of the TNF α -converting enzyme; BBG, Brilliant Blue G; BDNF, brain-derived neurotrophic factor; BzATP, 3'-O-(4-benzoyl)benzoyl adenosine 5'-triphosphate; CHPG, 2-chloro-5-hydroxyphenylglycine; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; Cx43 KO, Connexin 43 knockout mouse; Dep., dependent; DHPG, 3,5-dihydroxyphenylglycine; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid; EGTA, ethylene glycol tetraacetic acid; GYKI 52466, 1-(4-Aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine hydrochloride; HIV-1 gp120 IIIB, human immunodeficiency virus type 1 gp120(IIIB) protein; IA, Indoacetic acid; IP3 β , intracellular inositol 1,4,5-trisphosphate; L-644,711, ((5,6-dichloro-2,3,9,9a-tetrahydro-3-oxo-9a-propyl-1H-fluoren-7-yl)oxy)acetic acid; mAb 12G5, anti-chemokine (C-X-C motif) receptor 4 monoclonal antibody; MCPG, α -methyl-4-carboxyphenylglycine; NP-EGTA, o-nitrophenyl ethylene glycol tetraacetic acid; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; OxATP, adenosine 5'-triphosphate-2',3'-dialdehyde; pBPB, 4-bromophenacyl bromide; PD98059, 2'-amino-3'-methoxyflavone; PDC, 1-trans-pyrrolidine-2,4-dicarboxylate; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate; PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; SDF-1 α , stromal cell-derived factor-1 α ; SITS, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid; SNARE-domain, synaptobrevin 2 amino acids 1-96; Stg 4 siRNA, synaptotagmin 4 small interfering RNA; sTNFR1, soluble tumor necrosis factor-receptor 1; t-ACPD, trans-($\pm$)-1-amino-1,3-cyclopentanedicarboxylate; TBHA, L-threo- β -hydroxyaspartate; TBOA, DL-threo-beta-benzoyloxyaspartate; TNF α , tumor necrosis factor α ; U73122, 1-(6-(17 β -methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione.

* Connexin hemichannel opening is achieved in low extracellular Ca²⁺.