

Pathological Role for Exocytotic Glutamate Release from Astrocytes in Hepatic Encephalopathy

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Abstract: Liver failure can lead to generalized hyperammonemia, which is thought to be the underlying cause of hepatic encephalopathy. This neuropsychiatric syndrome is accompanied by functional changes of astrocytes. These glial cells enter ammonia-induced self-amplifying cycle characterized by brain oedema, oxidative and osmotic stress that causes modification of proteins and RNA. Consequently, protein expression and function are affected, including that of glutamine synthetase and plasmalemmal glutamate transporters, leading to glutamate excitotoxicity; Ca²⁺-dependent exocytotic glutamate release from astrocytes contributes to this extracellular glutamate overload.

Keywords: Astrocytes, exocytosis, glutamate release, hepatic encephalopathy.

HEPATIC ENCEPHALOPATHY AS TOXIC ASTROGLIOPATHOLOGY

Increase in the concentration of ammonia in the blood and in the brain, which result from several pathological conditions, such as urea cycle deficiencies, Reye's syndrome or different types of liver failure, causes polymorphic mental and behavioural manifestations such as confusion, forgetfulness, irritability, as well as alterations of consciousness represented by lethargy, somnolence and, in the terminal stages, coma associated with brain oedema that causes death [1, 2]. Hepatic encephalopathy (HE) resulting from an acute or chronic liver failure represents a substantial clinical problem (the acute liver failure being relatively rare affecting several thousands patients per year in the USA, whereas the chronic failure is diagnosed in ~ 5 million of people in the USA alone [1]). The deregulation of multiple neurotransmitter systems (glutamatergic, GABAergic, serotonergic, dopaminergic, etc.) lies at the very core of the HE-associated neuropsychiatric symptoms.

At the cellular level, the major changes in HE are detected in astrocytes, which include nuclear enlargement, peripheral margination of chromatin and prominent nucleoli [3, 4]. Enlarged astrocytes with watery swollen nuclei and cytosol (generally defined as Alzheimer's type II astrocytes; incidentally, the same pathologically remodelled cells are seen in autosomal recessive Wilson disease caused by abnormal copper metabolism) represent the main histopathological

hallmark of HE and hyperammonemia (HA). In contrast, there are little, if any, changes in neurones, indicating the major pathological role for astroglia. The second cellular substrate of HE is associated with microglia that undergo activation and contribute to neuro-inflammatory progression [5, 6].

The causal role of ammonia in hepatic encephalopathy was identified in 1930s [7] and it is now generally accepted that HE symptomatology results from an increase in brain concentration of ammonia to 3 - 5 mM [8] from the normal basal levels of ~ 0.2 - 3 μM [9]. In the brain, ammonia is detoxified by enzymatic conversion into glutamate or glutamine; these reactions being carried out by mitochondrial glutamate dehydrogenase (GDH) and cytosolic glutamine synthetase (GS), respectively [10]. The GS pathway (in which ammonium ion is used for the formation of glutamine from glutamate) is the most important and it is specifically associated with astroglial cells that exclusively express this enzyme [11] (Fig. 1). It should be noted that GDH reaction in the brain favors oxidative deamination of glutamate (and thus production of α-ketoglutarate) (Fig. 1) owing to the high NAD⁺/NADH ratio and the K_m for ammonia (14 - 26 mM) about four orders of magnitude higher than normal ammonia concentration [12, 13]. However, in HA, GDH could be an important mechanism for fixation of ammonia (and production of glutamate) as the normal activity of GS is saturated [14, 15] due to its K_m value for ammonia of 0.2 mM [16]. Hyperammonemia and HE conditions have been shown to activate cerebral GDH specifically towards glutamate oxidation [17] with further critical role for alanine aminotransferase mediated fixation of ammonia to alanine under excessive exposure to ammonia [15].

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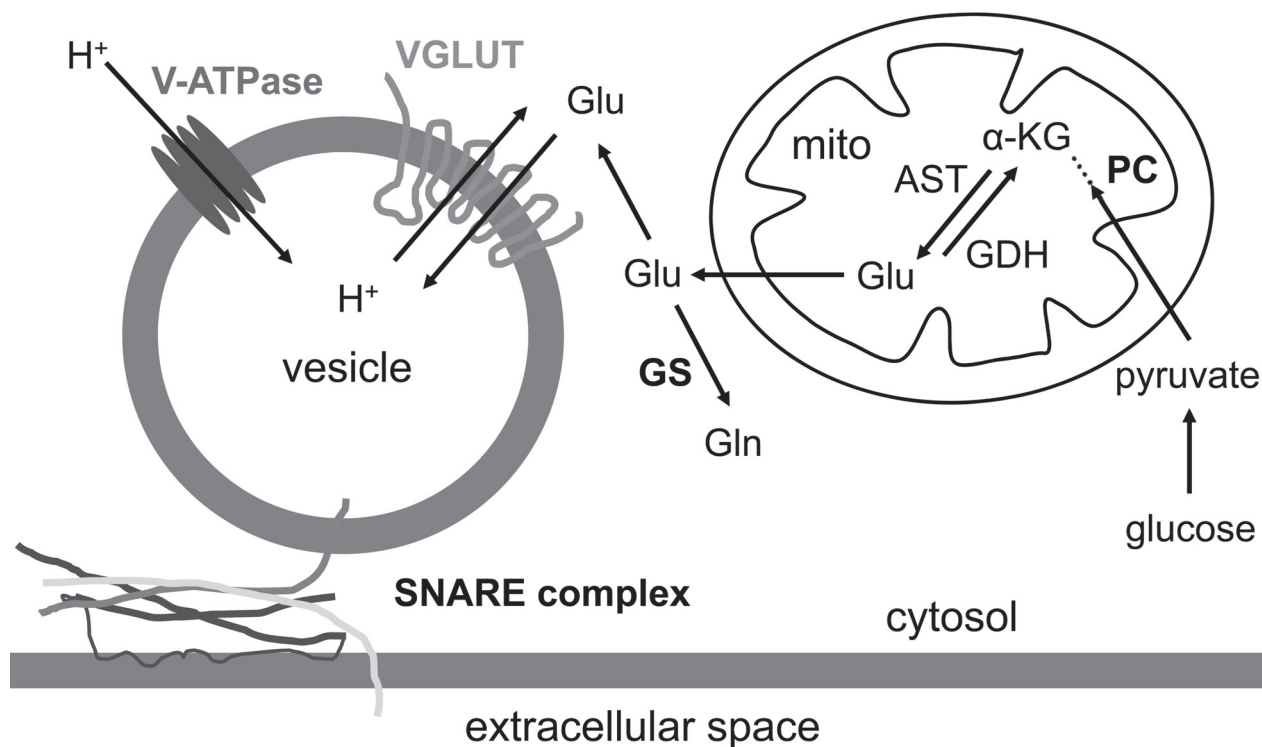


Fig. (1). Ca^{2+} -dependent glutamate release from astrocytes utilizes a vesicular, exocytotic pathway. Astrocytes express protein machinery necessary to execute exocytotic release of glutamate: the SNARE proteins synaptobrevin 2(Sb2)/cellubrevin (Cb), syntaxin 1, and SNAP23, along with vesicle filling proteins vesicular glutamate transporters (VGLUTs) isoforms 1-3 and vacuolar-type H^+ -ATPase (V-ATPase). Glutamate (Glu) can be synthesized in astrocytes *de novo* from pyruvate entry into the tricarboxylic acid cycle in mitochondria (mito) via pyruvate carboxylase (PC); glucose is broken down to pyruvate in the cytosol. Glutamate is converted from the cycle intermediate, α -ketoglutarate (α -KG), usually by transamination of aspartate via mitochondrial aspartate aminotransferase (AST). The synthesized glutamate once in the cytosol can then be converted to glutamine (Gln) by glutamine synthetase (GS), or transported into vesicles via vesicular glutamate transporters (VGLUTs). Glutamate dehydrogenase (GDH) normally generates α -KG by oxidative deamination. Drawing is not to scale. For simplicity syntaxin is shown truncated with its molecule portion N-terminal to the SNARE domain missing.

The glutamatergic neurotransmitter system is seemingly the most affected in HE. Acute injections of ammonia into the brain caused rapid death mediated through the overactivation of N-methyl-D-aspartate (NMDA) receptors, which could be prevented by NMDA receptors antagonists [5, 9]. Numerous experimental models of acute liver failure established a direct link between HE and increased brain glutamate load [18-20]. Glutamate concentration in the brain interstitium is defined by glutamate release and glutamate uptake. Glutamate release occurs through regulated exocytosis from presynaptic terminals and from astroglia; astrocytes also may release glutamate through several alternative pathways, such as the reversal of glutamate transporters (requires strong depolarisation in combination with cytosolic Na^+ and glutamate overloads; seems to operate only in extreme pathological conditions), glutamate exchange via the cystine/glutamate exchanger, diffusional release through volume- and Ca^{2+} -regulated anion channels, ionotropic purinergic receptors, connexon hemichannels or pannexins [21, 22]. Glutamate uptake is mediated mainly by astroglia specific glutamate transporters (classified as excitatory amino acid transporters EAAT1 and EAAT2 [23]). Concerted action of glutamate transports and cystine/glutamate exchanger defines glutamate concentration gradients between synaptic and perisynaptic compartments; EAATs keep very

low concentration of glutamate in the synaptic cleft thus facilitating neurotransmission, whereas cystine/glutamate exchanger sustains relatively high (10 - 20 μM) concentration of glutamate extrasynaptically thus maintaining tonic activation of metabotropic glutamate receptors [24]. Even slight deregulation of glutamate homeostasis may cause neuropsychiatric alterations as observed, for example, in schizophrenia or addictive disorders [24].

Astrocytes also act as the central element in glutamate metabolism and turnover. In addition to recycling glutamate through the tricarboxylic acid cycle (TCA) [25], astrocytes have the ability to synthesize glutamate *de novo* by virtue of the mitochondrial enzyme pyruvate carboxylase (Fig. 1); this enzyme is absent in neurones, which are therefore unable to synthesize glutamate *de novo* [26]; this enzyme is specifically expressed in astroglia [27]. In astrocytes glutamate is converted from the TCA intermediate α -ketoglutarate via transamination of another amino acid usually aspartate [25]; this reaction is carried by mitochondrial aspartate aminotransferase (Fig. 1). Astroglia based glutamate-glutamine shuttle is central for sustaining both glutamatergic and GABAergic transmissions (because GABA synthesis requires glutamate as the ultimate precursor). Glutamate, accumulated in astrocytes through the activity of

EAAT1/EAAT2 and the *de novo* synthesis, is converted to glutamine through GS; subsequently glutamine is transported back to glutamatergic and GABAergic terminals. In HE, the astroglial GS pathway is being “clogged” by excess of ammonia, and this affects astroglial glutamate accumulation and the glutamate-glutamine shuttle [28, 29] thus causing deregulated neurotransmission; in addition increase in astrocytic glutamine contributes to astrocyte swelling and promotes oxidative and osmotic stress [30]. Hyperammonemia and HE were also reported to decrease activity of pyruvate carboxylase in astrocytic mitochondria [31]. These changes activate glutamate release from astrocytes, which in turn amplifies excitotoxicity, oxidative stress and osmotic pressure on neural tissue. Protein nitration on tyrosines and RNA oxidation, due to generation of reactive oxygen and nitrogen species (ROS/RNOS), have been detected [32-34]. These several *circuli vitiosi* imposed on astrocytes by increased cerebral ammonia ultimately lead to glutamate overload, glutamate excitotoxicity and perturbed neurotransmission; these many pathogenic steps invoke psychiatric symptomatology and in severe cases the brain shut-down to the comatose state. In this review, we specifically analyse a single pathway of astroglial glutamate release associated with regulated vesicular exocytosis.

ASTROCYTES AND THE PROCESS OF REGULATED EXOCYTOSIS

Regulated exocytosis represents an evolutionary conserved system fundamentally important for many forms of intercellular communications and particularly employed in the synaptic transmission. Fast synaptic transmission in neuronal networks is governed by an increase in free calcium concentration ($[Ca^{2+}]_i$) in the presynaptic terminal triggered by an incoming action potential; this $[Ca^{2+}]_i$ increase, highly restricted in the spatial and temporal domains, initiates precisely synchronized discharge of vesicular cargo on a time scale of a few milliseconds. Astrocytes are also capable of executing the same process, although it is triggered by receptor-mediated and/or mechanically-elicited $[Ca^{2+}]_i$ elevation and develops on a time scale of milliseconds to seconds (for comparison of speed of exocytosis in various cells see [35]). Both neurones and astrocytes express protein machinery designed to support the exocytosis. Proteins indispensable for exocytosis have been identified in early 1990s [36] as components of the ternary soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, one of the most stable complexes, known to be resistant to SDS denaturation and temperatures up to 90 °C [37]. The crystal structure of the core ternary complex, identified in late 1990s [38], revealed specific interactions among three proteins: vesicular synaptobrevin 2 (Sb2, also referred to as vesicle associated membrane protein 2, VAMP2) with two plasma membrane target SNARE proteins, syntaxin 1A (Sx1A) and synaptosome-associated protein of 25 kD (SNAP25) (Fig. 1). These proteins form the α -helical bundle, i.e. coiled coils, consisting of four polypeptide coils, where Sb2 and Sx1A each contribute one coil, while SNAP25 contributes two. The SNAP25-containing ternary complex is mostly present in neurones, whereas in the majority of astrocytes the complex contains SNAP23 (see [39] for details and references). Another difference between

neuronal and astrocytic SNARE complexes is that astrocytes abundantly express cellubrevin (also known as VAMP3) [21, 40], which appears functionally interchangeable with Sb2 in Ca^{2+} -dependent exocytosis [41-43]. Despite the fact that the nature of protein-protein interactions within the complexes suggest strong hydrophobic interaction surrounding the electrostatic forces at a “zero” layer [38, 44] which similarly occur in both SNAP23- and SNAP25- containing ternary SNARE complexes, the presence of SNAP23 designates lower stability of the ternary complex [45]. This could contribute to some of the distinct characteristics of astrocytic and, in general, SNAP23-mediated exocytosis. Additionally, the differences between characteristics of exocytosis seen in astrocytes and neurones could arise from the actions of various associated proteins interacting with ternary complexes, such as complexins, Munc 13, Munc 18 or Ca^{2+} sensors synaptotagmins, which are also differentially expressed in these two major neural cell types. Nevertheless, the expression of common exocytotic proteins in astrocytes *in vitro*, in freshly isolated astrocytes, as well as astrocytes *in situ*, corroborates the existence of protein components essential for assembly of this membrane fusion device in astrocytes [reviewed in [39]].

Regardless of a cell type, the process of regulated exocytosis is dependent on a stimulus that induces an increase in $[Ca^{2+}]_i$, which then leads to vesicle fusion with the plasma membrane and release of transmitters. Astrocytes have been shown to release, through the process of regulated exocytosis, a number of neurotransmitters and neuromodulators, including glutamate [40], adenosine 5'-triphosphate (ATP) [46, 47], atrial natriuretic peptide [48], D-serine [49], tumour necrosis factor α [50], and brain derived neurotrophic factor [51].

In attempt to demonstrate that the mechanism of neurotransmitter release from astroglia occurs through the process of regulated exocytosis, a variety of experimental approaches have been utilised. Intracellular administration of Ca^{2+} chelators such as EGTA or BAPTA (delivered into the cytosol either by intracellular perfusion or by incubation with membrane permeable AM forms) effectively abolished astroglial exocytosis of neurotransmitters thus highlighting the regulatory role for Ca^{2+} ions [52, 53]. As mentioned above, the formation of the ternary SNARE complex precedes fusion of vesicles with the plasma membrane. Traditional approach to interfere with the complex assembly is to employ neurotoxins such as the tetanus toxin holoprotein, which light chain, once translocated to the cytosol, specifically cuts off a cytoplasmic portion of the uncomplexed Sb2 and/or cellubrevin thereby disabling Sb2/cellubrevin engagement with Sx1 and SNAP23/25 [21, 39, 54]. While the use of the holoprotein of neurotoxin is convenient in cell culture settings, *in vivo* experimentation requires different approaches. Therefore, the use of molecular genetics resulted in generation of transgenic mice with inducible expression of a soluble Sb2 cytoplasmic portion specifically in astrocytes. This dominant-negative SNARE domain (dnSNARE) competes with endogenous Sb2 for binding with Sx1 and SNAP23/25 thereby blocking the exocytotic release [55]. More recently, a transgenic mouse to block exocytosis by Cre recombinase-dependent cytosolic

expression of the botulinum neurotoxin serotype B light chain, which cleaves Sb2 and cellubrevin, was used to assess Müller cells gliotransmission in retina [56].

Based on the quantitative analysis of the relationship between $[Ca^{2+}]_i$ and regulated exocytosis [57, 58] it is likely that the calcium sensor in astrocytes is represented by one or more of synaptotagmins [21, 59]. Using flash photolysis of caged Ca^{2+} to control $[Ca^{2+}]_i$ in astrocytes when assessing consequential glutamate release electrophysiologically by either measuring currents in adjacent neurones, which acted as glutamate sensors [57], or monitoring changes in membrane capacitance (C_m) [58], the Hill coefficient for glutamate release of 2.1-2.7 and 5.3 was determined, respectively. Astrocytes express synaptotagmin 4, and the decrease of it by RNA interference, as well as expression of a mutated form, reduce Ca^{2+} -dependent glutamate release [60]. However, mammalian synaptotagmin 4 does not have calcium sensitivity [61] and the molecular identity of a calcium sensor in astrocytic exocytosis remains elusive.

Visualization of vesicles and their interactions with the plasma membrane during the exocytotic process in astrocytes became possible with the advent of total internal reflection microscopy (TIRFM), also referred to as evanescent wave microscopy, in combination with fluorescent dyes and optogenetic tools. Several groups utilized this technology to monitor vesicular exocytosis from astrocytes after stimulation [39, 62-65]. This experimental approach revealed that astrocytes, like neurones, display both modes of vesicle fusion: the full fusion, where the vesicle completely fuses with and collapses to the plasma membrane; and the transient fusion, where the vesicle transiently opens the fusion pore for substantially variable amount of time and then retrieves back. The nature of stimulation appeared to determine the type of fusion in astrocytes [39, 62].

REGULATED GLUTAMATE RELEASE FROM ASTROCYTES

The first evidence indicating that regulated exocytosis might be an underlying mechanism of glutamate release from astrocytes came from directly demonstrating that $[Ca^{2+}]_i$ elevation in individual astroglial cells was necessary and sufficient to cause glutamate release [66]. The $[Ca^{2+}]_i$ increase with subsequent glutamate release can be provoked by various stimuli including Ca^{2+} ionophores, photostimulation (using UV-A irradiation to increase non-selective cation permeability of the plasma membrane due to peroxidation of lipids/protein), photolysis of caged Ca^{2+} , mechanical stimulation, and chemical stimulation with various ligands, such as bradykinin, ATP and prostaglandins [21].

Exocytotic glutamate release, in addition to expressing the SNARE protein machinery and its associated proteins, requires operative proteins for filling vesicles with neurotransmitters. For glutamate these are represented by vacuolar-type H^+ -ATPase (V-ATPase) and vesicular glutamate transporter (VGLUTs) isoforms 1, 2 and 3, which are expressed in astrocytes [63, 67, 68]. The V-ATPase and VGLUTs work in synergy to concentrate glutamate inside vesicles. V-ATPase transports protons into vesicular lumen against their concentration gradient. This creates acidic

environment (pH ~ 5.5) within the vesicle. Since cytosolic pH is ~ 7.2, the electrochemical gradient created between the cytosol and the vesicular lumen is utilized by VGLUTs to deliver glutamate to the lumen, at expense of some proton loss into the cytosol (Fig. 1). While we do not know the exact ionic stoichiometry of this process, it appears that the chloride flux across the vesicular membrane, associated with the VGLUT operation, serves an important role in determining the relative magnitude of the electrochemical potential [69]. Functional capabilities of V-ATPase to acidify vesicles in cultured cortical astrocytes were demonstrated [67] by using an optogenetic tool, the chimeric protein synapto-pHluorin [70, 71]. The protein is a fusion of a pH-sensitive fluorescent protein, pHluorin, with the C-terminus of Sb2 which targets pHluorin into the vesicular lumen. Since pHluorin's fluorescence increases with alkalisation, vesicles in synapto-pHluorin transfected astrocytes brightened when the proton gradient is collapsed due to a treatment with bafilomycin A1, a blocker of V-ATPase known to induce alkalisation of the intravesicular lumen [67]. Bafilomycin A1 also blocked Ca^{2+} -dependent glutamate release from astrocytes [63, 67, 68, 72, 73]. Further evidence of vesicular acidification and glutamate co-dependence was presented in work using isolated Sb2-laden vesicles from astrocytes, which glutamate uptake was reduced in the presence of bafilomycin A1 [74].

Expression of all three isoforms of VGLUTs has been detected on glutamatergic vesicles both in neurone and astrocytes in cell culture and *in situ* [21, 39, 54]. Evidence for VGLUTs presence in astroglial cell bodies and processes derives from punctate labelling with fluorescent probes and immuno-gold labelling examined with electron microscopy [21, 39]. Furthermore, several groups have observed colocalization of VGLUTs with vesicular SNAREs Sb2 and cellubrevin [63, 67, 75]. Astrocytes possess functional VGLUTs, since the allosteric inhibitor of VGLUTs Rose Bengal blocked Ca^{2+} -dependent exocytotic glutamate release from astrocytes [67]. Over-expression of individual isoforms of VGLUTs in astrocytes showed that VGLUT-3, but not VGLUT-1 and VGLUT-2, enhances glutamate release from astrocytes without affecting their intracellular Ca^{2+} increase [76]. Moreover, inhibition of GS activity by L-methionine sulfoximine in astrocytes, which raises cytoplasmic glutamate levels [77], greatly increases the exocytotic glutamate release [76]. Thus, VGLUTs and cytoplasmic glutamate levels in astrocytes, in addition to cytosolic Ca^{2+} , can regulate exocytotic release from these cells.

Taken together, various independent laboratories presented ample evidence indicating astrocytic capabilities to release glutamate utilizing mechanism of regulated exocytosis in cell culture and *in situ* in physiological conditions. Next, we briefly touch upon effects that HE exerts on astrocytes in respect to glutamatergic gliotransmission *via* regulated exocytosis.

REGULATED GLUTAMATE RELEASE FROM ASTROCYTES IN HEPATIC ENCEPHALOPATHY

Since astrocytes play a central role in pathogenesis of HE, attempts were made to better understand mechanisms of ammonia-induced increase in extracellular brain glutamate concentrations in respect to glutamate release from these glial

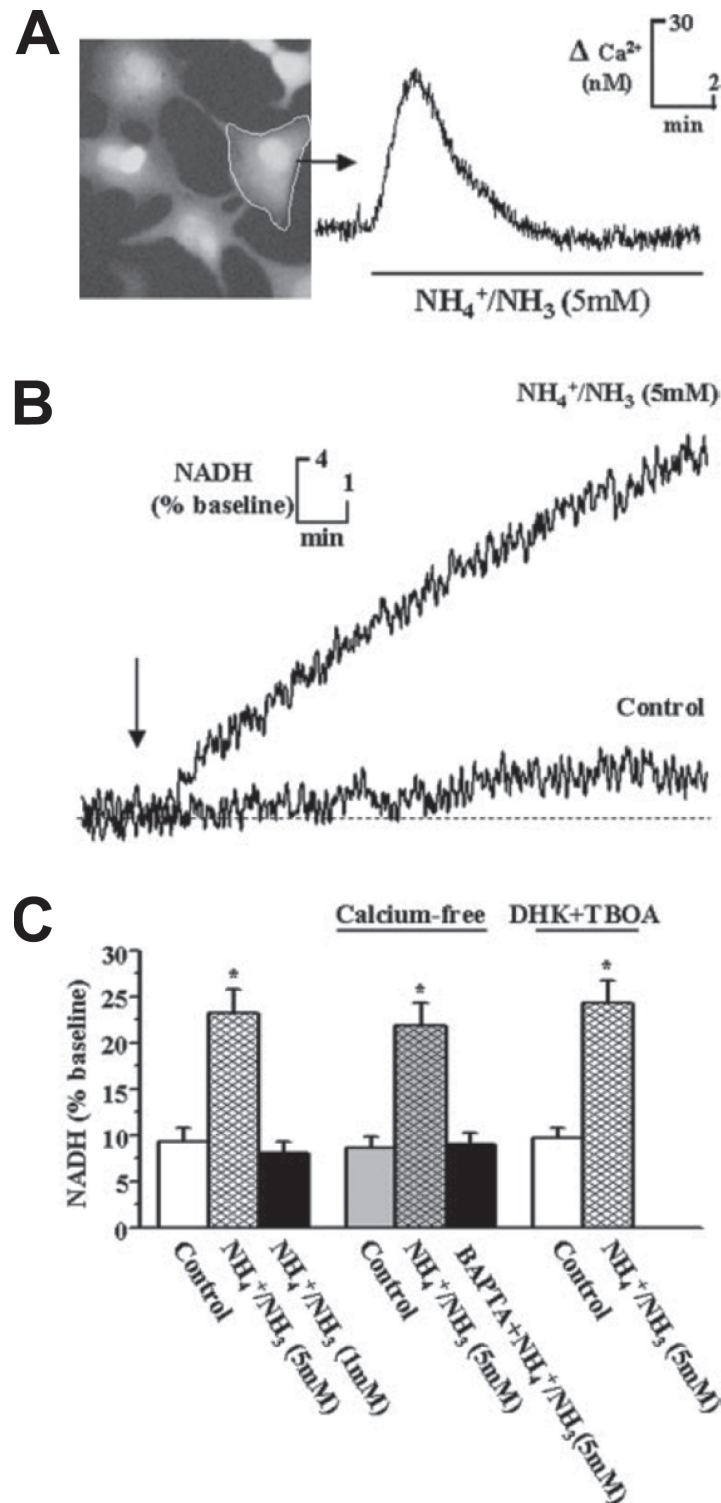


Fig. (2). Ammonia causes a transient increase in $[\text{Ca}^{2+}]_i$ and glutamate release from cultured astrocytes. A) Ammonia ($\text{NH}_4^+/\text{NH}_3$; 5 mM) induces a transient increase in $[\text{Ca}^{2+}]_i$ in cultured astrocytes. Image shows astrocytes loaded with a Ca^{2+} -sensitive fluorescent indicator. Time course of the response from the outlined astrocytes is shown in the trace. B) Addition of ammonia to cultured astrocytes (arrow) causes release of glutamate (recorded as an increase in the extracellular NADH fluorescence generate by an enzyme-linked assay). Untreated control does not show a substantial spontaneous accumulation of extracellular glutamate. C) Ammonia (5 mM, but not 1 mM)-induced glutamate release (reported by NADH fluorescence) from astrocytes is a Ca^{2+} -dependent manner. Incubation of astrocytes with BAPTA-AM, a membrane-permeable Ca^{2+} chelator, reduced the ammonia-induced glutamate release. EAAT-1 and EAAT-2 glutamate transporter inhibitors, DL-threo- β -benzyloxyaspartic acid (TBOA) and dihydrokainate (DHK), respectively, did not affect ammonia-induced glutamate release. *, statistically significant from control. Modified from [53].

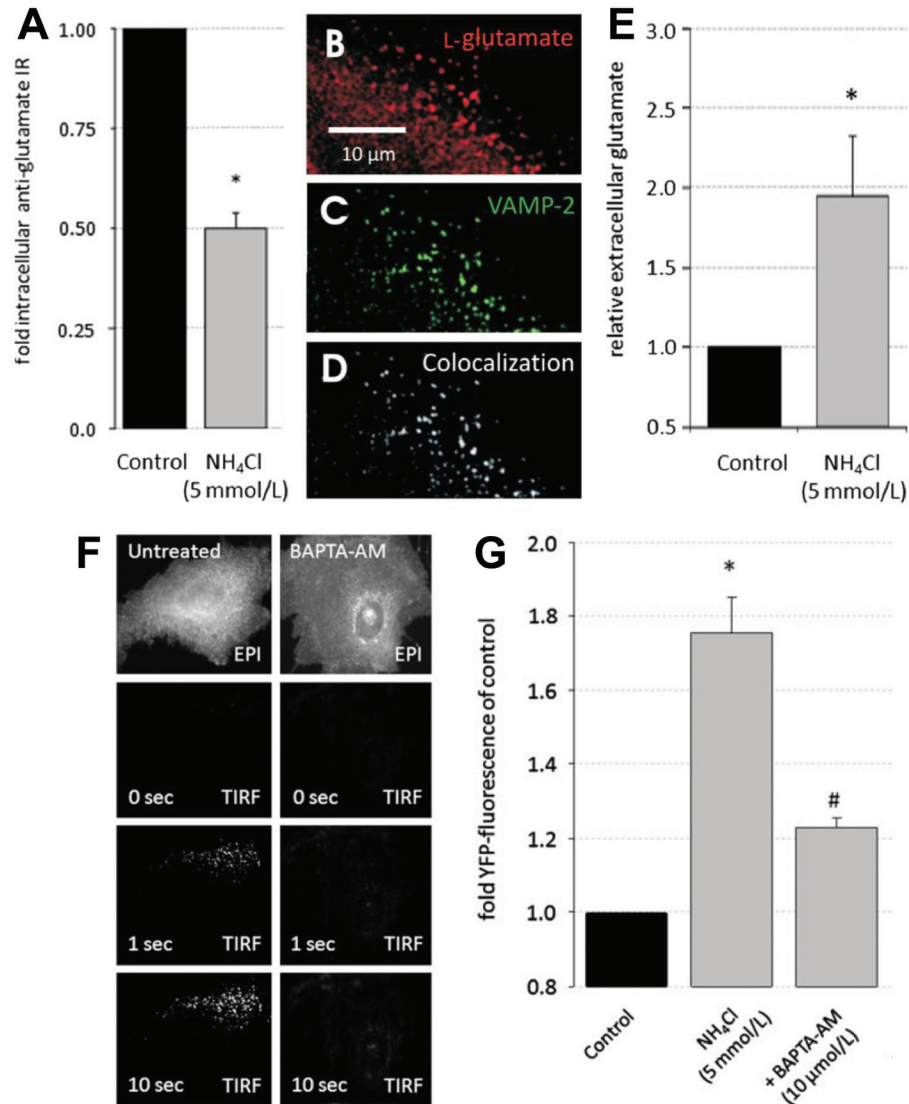


Fig. (3). Ammonia-elicited glutamate release from cultured astrocytes is mediated by vesicular pathway. A) Ammonia (5 mM NH_4Cl) reduces intracellular glutamate levels, based on anti-glutamate immunoreactivity (IR). B-D) Punctate anti-glutamate IR in astrocytes (B) colocalizes (D) with exogenously expressed VAMP2-YFP puncta/vesicles (C). E) Ammonia evokes release of glutamate from astrocytes into the extracellular space. F) Ammonia induces Ca^{2+} -dependent translocation of VAMP2-YFP to/near the plasma membrane of astrocytes. Epifluorescence (EPI) images show cultured astrocytes transfected to express VAMP2-YFP. The time lapse imaging (left vertical sequence) using TIRF microscopy reveals the ammonia-elicited rapid translocation of vesicles to the plasma membrane of astrocytes (time in lower left corner indicates exposure time to ammonia). Pre-incubation of astrocytes with the intercellular Ca^{2+} chelator BAPTA-AM abrogates this effect of ammonia (right vertical image sequence). G) Quantification of VAMP2-YFP fluorescence maxima measurements taken by TIRFM after the treatment with ammonia for 60 seconds. *and #, statistically significant from control and NH_4Cl treated astrocyte, respectively. Modified from [77].

cells. It was previously shown that an increase in the intracellular pH (alkalinisation) provokes an increase in $[\text{Ca}^{2+}]_i$ in various cells [78, 79]. Consequently, Rose *et al.* [53] hypothesized that Ca^{2+} -induced glutamate release could be one of the mechanisms and a contributing source for accumulation of extracellular glutamate. They exposed cultured and Ca^{2+} indicator-loaded astrocytes to NH_4Cl (referred to subsequently and interchangeably as ammonia or $\text{NH}_4^+/\text{NH}_3$) at a concentration (5 mM) relevant for acute and chronic liver failures (Fig. 2A, left-image). An addition of

extracellular NH_4Cl makes all the content of the cell alkaline because ammonia (NH_3) in equilibrium with ammonium ions (NH_4^+) in solution diffuses across cell membranes and increases the pH [78, 79]. Ammonia induced a transient Ca^{2+} response in astrocytes (Fig. 2A, right-trace), which was attributed to Ca^{2+} release from the internal stores, as the ammonia-induced Ca^{2+} responses were similar in the absence of extracellular Ca^{2+} ; thapsigargin, an inhibitor of the endoplasmic store Ca^{2+} -ATPase corroborated this finding. Application of $\text{NH}_4^+/\text{NH}_3$ also triggered glutamate release

from astrocytes (Fig. 2B), which was Ca^{2+} -dependent, since astrocytes loaded with BAPTA-AM (a cell-permeant form of this Ca^{2+} chelator) and bathed in the extracellular saline free of Ca^{2+} show repealed ammonia-induced glutamate release (Fig. 2C). Of note, glutamate measurements were based on an enzyme-linked assay, whereby glutamate release is reported as the extracellular accumulation of NADH, an assay/reaction product that is fluorescently detected. The reversal of plasmalemmal glutamate transporters did not play a role in ammonia-induced Ca^{2+} -dependent glutamate release, since their pharmacological inhibition did not affect the release (Fig. 2C). Taken together, an increase in extracellular ammonia triggers a cytosolic alkaline shift that causes the Ca^{2+} -dependent glutamate release [53]. Although no direct evidence was presented (but see below), authors speculated that the mechanism of the release was exocytotic since recorded rises in $[\text{Ca}^{2+}]_i$ were otherwise sufficiently high to trigger Ca^{2+} -dependent glutamate release from astrocytes, as per threshold levels for this process determined elsewhere [57]. They further estimated that *in situ*, the amounts of released glutamate would be in the millimolar range, a concentration more than adequate to cause hyperexcitability of nearby neurones due to overstimulation of NMDA receptors [53].

A subsequent study defined that ammonia-induced glutamate release occurs *via* vesicular recruitment [77]. In parallel assessment of intra- and extracellular glutamate levels (by indirect immunocytochemistry and an enzyme-linked assay, respectively) indicated that NH_4Cl (5 mM) causes a reduction of intracellular glutamate in astrocytes (Fig. 3A), while observing an increase in the extracellular level of glutamate (Fig. 3E); it should be noted that a hyposmotic medium also caused a robust glutamate release from astrocytes. Nonetheless, glutamate in astrocytes is packaged in vesicles that contain VGLUTs and Sb2; synapto-pHluorin expressing vesicles in ~ 86% of cases colocalized with VGLUT1 immunoreactivity [80]. Indeed, Gorg *et al* [77] reported that in astrocytes glutamate itself colocalizes with exogenously expressed VAMP2-YFP chimera (Fig. 3C-D), in which VAMP2 is appended at its intravesicular C-terminus with yellow fluorescence protein (having a reduced pH sensitivity when compared to pHluorin). Consequently, they used VAMP2-YFP and total internal reflection fluorescence microscopy (TIRFM) to study whether ammonia-induced glutamate release is accompanied by translocation of vesicles to the plasma membrane. Namely, TIRFM provides an excellent method for studying fluorescently tagged molecules at and/or very near to the plasma membrane in live astrocytes [62, 81]. Ammonia induced translocation of VAMP2-YFP-laden vesicles to/near the plasma membrane of astrocytes (Fig. 3F, G). This effect was abolished by a pretreatment of astrocytes with BAPTA-AM (Fig. 3F, G), indicating Ca^{2+} dependency of this process. Taken together, the work from two different groups points to Ca^{2+} -dependent exocytosis as a credible mechanism underlying ammonia-induced glutamate release from astrocytes.

CONCLUDING REMARKS AND FUTURE ENDEAVOURS

The purpose of this essay was to summarize up-to-date knowledge on how exocytotic glutamate release from

astrocytes gets affected when tightly regulated brain homeostasis is severely upset in HA that occurs in HE. The ammonia-evoked glutamate release is likely not only regulated by cytosolic Ca^{2+} , but rather should also depend on metabolic pathways. In particular, as the astroglial GS pathway gets utilized to its saturation, there is an increased production of not only glutamine, but also glutamate. An increase in the cytosolic glutamate levels leads to an increased packaging of this transmitter into astrocytic secretory vesicles resulting in an enhancement of glutamatergic gliotransmission [76]; although this type of regulation is expected to occur in HA/HE, it has not been experimentally demonstrated, yet. Additional consideration should be given to an increase in astrocytic intracellular glutamine levels contributing to astrocyte swelling, which itself can lead to release of various osmolytes, including glutamate, through volume-regulated anion channels (VRACs). Whether this astrocytic glutamate release conduit utilizes SWELL1, a recently identified plasmalemmal protein as an essential VRAC component [82], awaits future experimentation.

There is evidence that receptor-mediated intracellular Ca^{2+} increases in astrocytes, elicited by ATP, can result in transient cell swelling leading to glutamate release through VRACs [83]; not only that anion channel blockers impaired this release, but, unexpectedly, so did the intracellular presence of BAPTA. Although two compounds known to affect vesicular release, tetanus toxin and bafilomycin A1, did not prevent swelling-induced glutamate release, the observation that a facet of this secretory mechanism appears to be Ca^{2+} -sensitive raises the question of how swelling-induced and vesicular glutamate release pathways in astrocytes cross-talk. Of course, the fact that purinergic stimulation induces astrocytic swelling is also intriguing, since astrocytes can release ATP using exocytosis along with several other mechanisms [84]. It is plausible that there might be an interface between glutamatergic and purinergic systems in mediating effects that the liver failure exerts on the brain. Of note, there is a reduction of ATP levels associated with a prolonged ammonia treatment of astroglial cultures [85], and a fall in brain ATP levels that accompany severe encephalopathy in portacaval-shunted rats [86].

The relatively recent discovery of the presence of vesicular release proteins in astrocytes resulted not only in the need to reinterpret previous findings in relation to neurone-astrocyte bi-directional signaling, but also of the role of exocytosis in HA and HE, as already discussed. Another recent major finding that can similarly recontextualize our perception of the brain operation in health and disease is that of the presence of functional NMDA receptors on astrocytes [87-89]. Stimulation of astroglial NMDA receptors have been shown to increase astrocytic production of kynurenic acid, the endogenous inhibitor of $\alpha 7$ nicotinic and NMDA receptors [90]; kynurenic acid is considered to contribute to neurotransmitter imbalance and psychotic symptoms in schizophrenia [91] and may also be involved in psychotic component of HE. Rapid death due to acute injections of ammonia into the brain can be prevented by NMDA receptors antagonists. Similarly, the NMDA receptor antagonist memantine attenuated HE severity in experimental

acute liver failure [92]. These findings pose a question of the cell-type site of NMDA receptor blockers beneficial effects, i.e., whether actions were arbitrated by neuronal and/or astroglial NMDA receptors. As astroglial NMDA receptors lack the magnesium block and hence operate at resting membrane potentials [88], they could be a prime suspect in the aetiology of HE, and may offer a fertile site for novel medical intervention in this pathological condition.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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