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ORIGINAL PAPER

Effective Mechanism for Synthesis of Neurotransmitter Glutamate and its Loading into Synaptic Vesicles

Kouji Takeda[1,4] · Tetsufumi Ueda[1,2,3]

Abstract Glutamate accumulation into synaptic vesicles is a pivotal step in glutamate transmission. This process is achieved by a vesicular glutamate transporter (VGLUT) coupled to v-type proton ATPase. Normal synaptic transmission, in particular during intensive neuronal firing, would demand rapid transmitter re-filling of emptied synaptic vesicles. We have previously shown that isolated synaptic vesicles are capable of synthesizing glutamate from α-ketoglutarate (not from glutamine) by vesicle-bound aspartate aminotransferase for immediate uptake, in addition to ATP required for uptake by vesicle-bound glycolytic enzymes. This suggests that local synthesis of these substances, essential for glutamate transmission, could occur at the synaptic vesicle. Here we provide evidence that synaptosomes (pinched-off nerve terminals) also accumulate α-ketoglutarate-derived glutamate into synaptic vesicles within, at the expense of ATP generated through glycolysis. Glutamine-derived glutamate is also accumulated into synaptic vesicles in synaptosomes. The underlying mechanism is discussed. It is suggested that local synthesis of both glutamate and ATP at the presynaptic synaptic vesicle would represent an efficient mechanism for swift glutamate loading into synaptic vesicles, supporting maintenance of normal synaptic transmission.

Keywords Synaptic vesicles · Aspartate aminotransferase · Glutamate · VGLUT · Local synthesis · Efficient mechanism

Abbreviations
AAT	Aspartate aminotransferase
ACPD	1-Aminocyclopentane-1,3-dicarboxylic acid
α-KGA	α-Ketoglutarate
FCCP	Carbonyl cyanide p-(trifluoromethoxy)-phenylhydrazone
GAPDH	Glyceraldehyde-3-phophate dehydrogenase
GABA	γ-Aminobutyric acid
GDH	Glutamate dehydrogenase
Gln	Glutamine
GOT	Glutamate oxaloacetate aminotransferase
HPLC	High pressure liquid chromatography
hsp	Heat shock protein
SV	Synaptic vesicle
TCA	Tricarboxylic acid
VGLUT	Vesicular glutamate transporter

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Introduction

In the central nervous system, the majority of neural communication involves glutamate transmission. In glutamate transmission, glutamate loading into synaptic vesicles is an initial pivotal step, the gate for glutamate to enter the committed neurotransmitter pathway, away from the metabolic pathway [1–6]. This process enables synaptic vesicles to sufficiently concentrate glutamate prior to its release to the synaptic

cleft. Glutamate thus concentrated, when released, serves as a signal to fire and regulate postsynaptic and presynaptic neurons via activation of its various receptors (AMPA-, kainate-, NMDA-preferring types, and the metabolic type).

Glutamate accumulation into synaptic vesicles is achieved by an energy-dependent, glutamate-specific vesicular uptake system, consisting of v-type proton-pump ATPase and a vesicular glutamate transporter (VGLUT) [1, 3, 6–18]. Vesicular proton-pump ATPase generates an electrochemical proton gradient, which is harnessed by VGLUT. Three isoforms (VGLUT1-3, *SLC17A7, SLC17A6* and *SLC17A8*, respectively) are known [14, 15, 19–30], VGLUT1 being the major. They have different brain regional, cellular, and subcellular distributions. They are expressed not only in the brain and spinal cord, but also in "non-nervous tissues." Gaged by their distributions, they perform different physiological functions [6]. However, they exhibit no significant difference in biochemical uptake function [18, 19, 23, 25, 27, 31]. The functional difference between VGLUT1 and VGLUT2 is thought to lie in membrane trafficking and transmitter release probability [19, 23, 28, 29]. VGLUT1 appears to have a role in regulating release probability [19, 23, 28, 29, 32, 33] and in synaptic plasticity [23, 34, 35]. The low release probability associated with VGLUT1 was shown due to its binding to endophilin A1 [32]. More recent studies however indicate that vesicular glutamate concentration itself affects release probability [33].

Synaptic vesicles are endowed with glycolytic ATP-generating enzymes, glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglycerate kinase complex and pyruvate kinase, both capable of fueling VGLUT [36, 37]. Evidence suggests that vesicular glutamate loading in the presynaptic nerve ending is largely achieved at the expense of glycolytically produced ATP [36]. These observations suggest that local synthesis of ATP at the synaptic site plays an important role in accumulation of glutamate into synaptic vesicles. This could provide an efficient mechanism for vesicular glutamate loading into the nerve terminal, and hence in glutamate transmission.

In an effort to extend the local synthesis concept to glutamate synthesis, we have explored the possibility that synaptic vesicles could be capable of producing the VGLUT substrate. In eukaryotes, glutamate can be formed from α-ketoglutarate by glutamate dehydrogenase (GDH) or by aspartate aminotransferases (AAT), as well as from glutamine by glutaminase. Takeda et al. [38] have shown that isolated synaptic vesicles have the capacity to produce glutamate from α-ketoglutarate (α-KGA) by AAT, for immediate uptake by VGLUT. In this study, we provide evidence supporting the notion that α-KGA-derived glutamate, produced by AAT, is accumulated into synaptic vesicles within the nerve ending. The significance of these observations is discussed.

Materials and Methods

Animals

Sprague Dawley rats (180–220 g) were obtained from Charles River. All animal procedures were approved by the University of Michigan Committee for Use and Care of Animals (UCUCA) in accordance with National Institutes of Health Guide for the Use and Care of Laboratory Animals. Rats were sacrificed by decapitation, and cerebrum extracted.

Chemicals

$[^{14}C]$α-KGA, $[^3H]$glutamine, and $[^3H]$glutamate were purchased from PerkinElmer, and bafilomycin from Fisher Scientific. All other chemicals were from Sigma-Aldrich.

Assay for Synaptosomal Uptake of Radioactive Ligands and Vesicular Radioactive Content

Crude synaptosomes (P2 fraction: 214–263 μg of protein) prepared from calf frontal cortex (obtained from a local slaughterhouse) were suspended in 200 μl oxygenated (95% O_2–5% CO_2) Krebs–Ringer buffer containing 171.5 mM NaCl, 1.84 mM K_2HPO_4, 2.45 mM $MgSO_4$, 1.59 mM $CaCl_2$, 6.13 mM glucose and 12.25 mM HEPES-NaOH (pH 7.4), and preincubated at 37 °C for 10 min in the absence or presence of 5 μM bafilomycin. After addition of 53 μM $[^{14}C]$α-KGA (56.8 mCi/mmol), $[^3H]$glutamine (94.7 or 190 mCi/mmol), or $[^3H]$glutamate (190 mCi/mmol), synaptosomes were incubated for 30 min. Aliquots (40 μl) were removed and filtered on Whatman GF/C filters to determine the total amount of $[^{14}C]$α-KGA, $[^3H]$glutamine, or $[^3H]$glutamate taken up by the synaptosomes, and the rest immediately frozen on dry ice. For determination of vesicular radioactive contents, the frozen synaptosomes (160 μl) were thawed, followed by addition of 1.5 ml of ice-cold hypotonic solution containing 6 mM Tris–maleate (pH 8.1) and 2 mM aspartate. The mixture was allowed to stand for 20 min on ice, and centrifuged at 4 °C for 10 min (Beckman microfuge, 17,600g_{max}). The supernatant (1.2 ml) was filtered on Whatman GF/C filters, and radioactivity retained on filter determined in a Beckman LS 6500 scintillation spectrophotometer.

Analysis of Radioactive Compounds Accumulated into the Synaptic Vesicle Fraction from Synaptosomes Which Have Taken Up $[^{14}C]$α-KGA or $[^3H]$Glutamine

The calf cerebral cortex P2 fraction was incubated with $[^{14}C]$α-KGA or $[^3H]$glutamine in 200 ml of Krebs-Ringer solution, followed by addition of 1.6 ml of ice-cold

hypotonic solution containing 6 mM Tris–maleate (pH 8.1) and 2 mM aspartate, as described above. The crude synaptic vesicle fraction (the material retained on the filter) was collected, as described above, and subjected to 80 % ethanol extraction and analysis for radioactive compounds by HPLC, as previously described [37].

Purification of Synaptosomes

In some experiments (Fig. 2), crude synaptosomes (P2 fraction) in 0.32 M sucrose, 0.25 mM DTT, and 1 mM EDTA were subjected to Percoll-gradient centrifugation, as described by Dunkley et al. [39]. Two aliquots (3 ml each) of the P2 fraction derived from 10 g of fresh calf frontal cortex were layered on top of the gradient consisting of 3, 10, 15, and 23 % Percoll (4 ml each per Sorvall SS-34 rotor tube) and centrifuged at 20,000 rpm (47,800g_{max}) for 5 min. Fractions 1–4 represent the gradient interfaces from top to bottom, respectively. Fraction 5 represents the pellet. Each fraction (collected from two tubes) was suspended in 20 ml of Krebs-Ringer buffer containing 140 mM NaCl, 1.5 mM K_2HPO_4, 2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 5 mM glucose and 10 mM HEPES–NaOH (pH 7.4) and centrifuged at 14,000 rpm (23,400g_{max}) for 10 min, and resulting pellets suspended in Krebs-Ringer buffer.

Results

Radioactive Material Derived from [^{14}C]α-Ketoglutarate is Accumulated into Synaptic Vesicles in Synaptosomes as is that Derived from [^{14}H]Glutamine

We have previously shown that isolated synaptic vesicles are associated with AAT that catalyzes the synthesis of glutamate from α-KGA for its immediate uptake [38]. In an effort to demonstrate that this could occur *in the intra-nerve ending* milieu, synaptosomes (pinched-off nerve terminals) were incubated with [^{14}C]α-KGA in an oxygenated Krebs-Ringer solution in the absence or presence of the proton-pump ATPase inhibitor bafilomycin, and the amount of radioactive material incorporated into the synaptic vesicle fraction determined. Bafilomycin (1 μM) has previously been shown to abolish glutamate uptake into isolated synaptic vesicles [10]. Figure 1 shows that although bafilomycin (5 μM) had negligible effect on total uptake of [^{14}C]α-KGA into synaptosomes (A), it led to markedly reduced accumulation of radioactive material into synaptic vesicles in synaptosomes (B). The majority of radioactive material accumulated in synaptic vesicles was determined to be glutamate (Fig. 2a). This is compatible with the observation by Shank and Campbell (40) that α-ketoglutarate taken up into
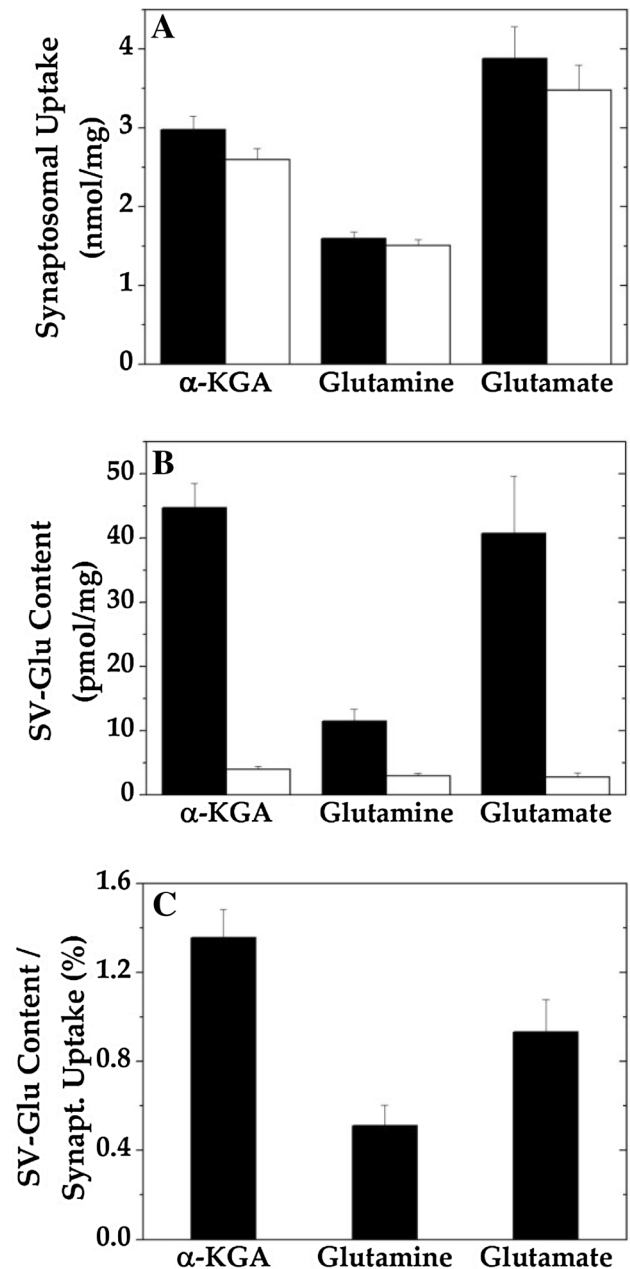

Fig. 1 α-KGA-derived glutamate uptake into synaptic vesicles occurs in synaptosomes, as does glutamate uptake derived from glutamine and exogenous glutamate. Synaptosomes (214–263 μg of protein) were preincubated in the absence (*black bars*) or presence (*white bars*) of bafilomycin, followed by incubation with [^{14}C]α-KGA, [^{3}H]glutamine, or [^{3}H]glutamate, and vesicular content of radioactive material (glutamate) derived from each (**b**), as well as for synaptosomal uptake of each (**a**), was determined, as described in "Materials and Methods" section. For calculation of the ratio of SV-Glu content/synaptosomal uptake (**c**), vesicular glutamate content values obtained in the presence of bafilomcin were corrected for the inhibitory effect of bafilomcin on synaptosomal uptake and subtracted from those obtained in the absence of bafilomycin (**b**), and the bafilomycin-sensitive vesicular content value was divided by total synaptosomal uptake value in the absence of bafilomycin (**a**). Data on experiments with α-KGA and glutamine are the mean ± SEM from seven independent experiments and, for exogenous glutamate, the mean ± SEM from four independent experiments

synaptosomes is mainly converted to glutamate. In addition, radioactive material produced from α-KGA by, and accumulated into, isolated synaptic vesicles, is essentially all glutamate, and α-KGA itself (i.e., in the absence of aspartate) is not taken up into synaptic vesicles [38]. Moreover, glutamate has been shown to represent the major neurotransmitter taken up into highly purified synaptic vesicles in an energy-dependent manner [40], consistent with glutamate being the principal neurotransmitter in brain [41].

Bafilomycin-sensitive incorporation of radioactive material was considered to reflect the vesicular content of largely glutamate. Taking into account the small effect of bafilomycin on synaptosomal uptake, the vesicular accumulation of glutamate was expressed as the ratio (Fig. 1c) of bafilomycin-sensitive vesicular content (Fig. 1b) to synaptosomal uptake (Fig. 1a). Thus, Fig. 1 indicates that [14C]glutamate derived from [14C]α-KGA in synaptosomes is accumulated into synaptic vesicles.

Since glutamine is generally thought to be the principal precursor of the neurotransmitter glutamate, we have also measured vesicular content of radioactive material derived from [3H]glutamine in synaptosomes to compare with that derived from [14C]α-KGA. Figure 1 also shows that vesicular accumulation of radioactive material derived from [3H]glutamine is bafilomycin-sensitive. The majority of the radioactive substance accumulated is glutamate (Fig. 2b).
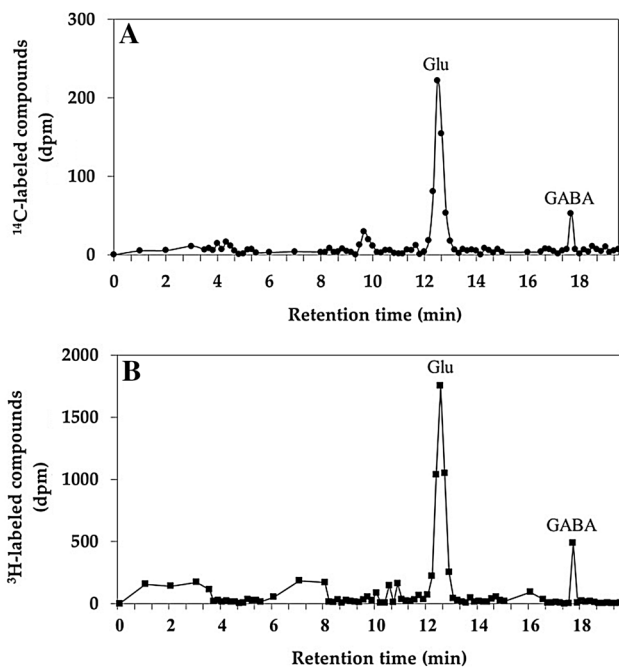

Fig. 2 HPLC chromatogram of [14C]α-KGA and [3H]Gln-derived products accumulated into synaptic vesicles in synaptosomes. Crude synaptosomes (P2 fraction) were incubated with [14C]α-KGA (**a**) or [3H]Gln (**b**), and the synaptic vesicle fraction was collected and subjected to analysis for radioactive compounds by HPLC, as described in "Materials and Methods" section

The metabolic product of glutamine taken up into synaptosomes has also been shown to be largely glutamate [42]. Of interest, Fig. 1 indicates that α-KGA is more effective than glutamine as a precursor of glutamate in synaptosomes from calf frontal cortex. Surprisingly, when compared with vesicular [3H]glutamate accumulation in synaptosomes exogenously loaded with [3H]glutamate, vesicular accumulation of α-KGA-derived putative glutamate appears at least as efficient. This suggests that conversion of α-KGA to glutamate is rapid in synaptosomes, consistent with the high rates of AAT and the malate/aspartate shuttle in synaptosomes [43].

In order to confirm that vesicular accumulation of α-KGA- and glutamine-derived glutamate indeed occurs in synaptosomes, the crude synaptosome preparation was purified by Percoll-gradient centrifugation [39] and various fractions analyzed for vesicular content of glutamate derived from α-KGA and glutamine. Figure 3 shows that vesicular accumulation of glutamate derived from both α-KGA and glutamine is most abundant in the fraction richest in synaptosomes (fraction 4).

Vesicular α-KGA- and glutamine-derived glutamate accumulation in synaptosomes occurs in a time-dependent and bafilomycin-sensitive manner (Fig. 4). Bafilomycin greatly decreased vesicular accumulation of α-KGA- and glutamine-derived glutamate at all incubation times. Bafilomycin-sensitive vesicular accumulation of glutamate derived from α-KGA was 2.5- to threefold faster than that of glutamate derived from glutamine. This is likely due to the multi steps involved in generating glutamine-derived α-KGA available for extra-mitochondrial AAT: metabolism of glutamine, first to glutamate by mitochondrial glutaminase, then to α-KGA by mitochondrial AAT and GDH, followed by translocation of α-KGA to the cytoplasm. In addition, not all the α-KGA produced from glutamine may be transported out of mitochondria; part could be metabolized via the TCA cycle.

Vesicular Loading of α-KGA-Derived Glutamate in Synaptosomes is Mediated by AAT and Fueled by Glycolytic ATP

In an effort to show that α-KGA-derived glutamate accumulation into synaptic vesicles in synaptosomes is mediated by AAT, we have examined the effect of hydroxylamine, a membrane-permeable AAT inhibitor, on that process. As shown in Fig. 5, hydroxylamine resulted in decreased vesicular content of α-KGA-derived "glutamate" in a dose-dependent manner, confirming this supposition.

Evidence suggests that glutamate uptake into vesicles in synaptosomes is harnessed largely by glycolysis-generated ATP, a minor source of cellular energy [36]. In order to see whether this also applies to vesicular uptake of
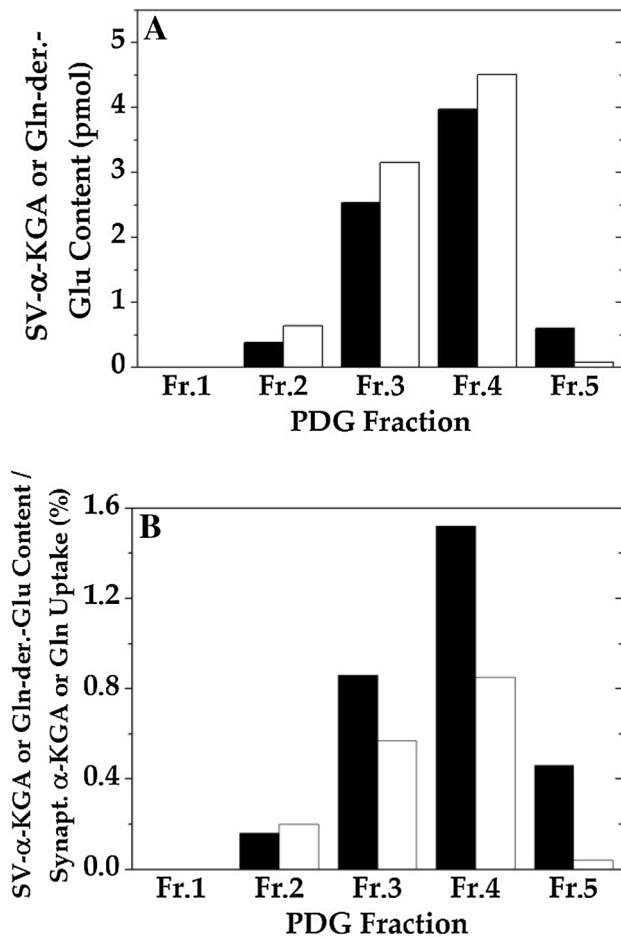
Fig. 3 Vesicular accumulation of [^{14}C]α-KGA- and [^{3}H]glutamine-derived glutamate in various fractions obtained upon Percoll-gradient centrifugation of the crude synaptosome (P2) fraction. An aliquot of each fraction, which had been preincubated in the absence or presence of bafilomycin, was assayed for synaptosomal uptake of [^{14}C]α-KGA and [^{3}H]glutamine and for vesicular content of [^{14}C]α-KGA-derived glutamate (*black bars*) and [^{3}H]glutamine-derived glutamate (*white bars*), as described in "Materials and Methods" section, except that the incubation medium contained 53 μM [^{14}C]α-KGA *plus* 53 μM [^{3}H]glutamine (double-labeling). Fractions 1–4 denote the gradient interfaces from top to bottom, respectively. Fraction 5 denotes the pellet. Vesicular glutamate content (**a**) represents bafilomycin-sensitive vesicular glutamate content and expressed as the ratio (**b**) of vesicular glutamate content/synaptosomal uptake. *PDG* Percoll-density gradient

α-KGA-derived glutamate, we have examined the effect of the GAPDH inhibitor iodoacetate on this process and compared it with that of the mitochondrial ATP synthase inhibitor oligomycin. As shown in Fig. 6, vesicular accumulation of α-KGA-derived glutamate is substantially reduced in the presence of iodoacetate but little affected by oligomycin. On the other hand, overall ATP levels in synaptosomes are decreased in a similar fashion under these conditions [36]. These observations suggest that glycolytically produced ATP plays a major role in fueling vesicular loading of α-KGA-derived glutamate as well.

Vesicular Loading of α-KGA-Derived Glutamate is Increased by Depolarization, as is that of Glutamine-Derived Glutamate

Memory formation involves high frequency neuronal firing, which leads to neuronal membrane depolarization. In order to examine whether vesicular loading of α-KGA- and/or glutamine-derived glutamate is modulated by neuronal activity, synaptosomes were depolarized and vesicular content of α-KGA- and glutamine-derived glutamate determined. In this experiment, we used synaptosomes prepared from the fresh cerebrum of matured rats. Depolarization inhibited synaptosomal uptake of both α-KGA and glutamine (Fig. 7a), as expected. Nonetheless, the measured amount of α-KGA-derived glutamate accumulated in synaptic vesicle was hardly changed (Fig. 7b). When the latter was corrected for the reduced uptake into synaptosomes, vesicular content (per amount of α-KGA taken up into synaptosomes) of both α-KGA- and glutamine-derived glutamate was actually increased (Fig. 7c). This indicates that while depolarization suppresses uptake into synaptosomes of α-KGA and glutamine, it leads to enhanced vesicular loading of glutamate derived from α-KGA and glutamine, perhaps in order to maintain vesicular glutamate content homeostasis.

It is noted that in this experiment, which employed synaptosomes prepared from fresh rat cerebrum, that the vesicular content of glutamate derived from α-KGA is lower than that derived from glutamine. The reason is not known. This could be due to the difference in brain region, species, or developmental stages.

Discussion

Synaptic Vesicles Have the Capacity to Synthesize the VGLUT Substrate Glutamate

We have provided evidence that synaptic vesicles are capable of generating the neurotransmitter glutamate from α-KGA with aspartate as the amino donor, providing the VGLUT substrate for immediate uptake into vesicles, and resulting in an increased vesicular concentration of glutamate [38]. The vesicular concentration step is a process essential for glutamate to function as a neurotransmitter signal. We propose that this glutamate synthesis is efficiently achieved in large part by vesicle-bound AAT, not by glutaminase. This vesicle-bound aminotransferase is highly specific to L-aspartate, and its affinity for aspartate ($K_m = 0.9$ mM) is similar to that of mitochondrial AAT, but substantially higher than that of cytosolic AAT ($K_m = 6.7$ mM) [44]. Not only in kinetic properties but also in immunoreactivity, vesicle-bound AAT is distinct from cytosolic AAT and indistinguishable from mitochondrial
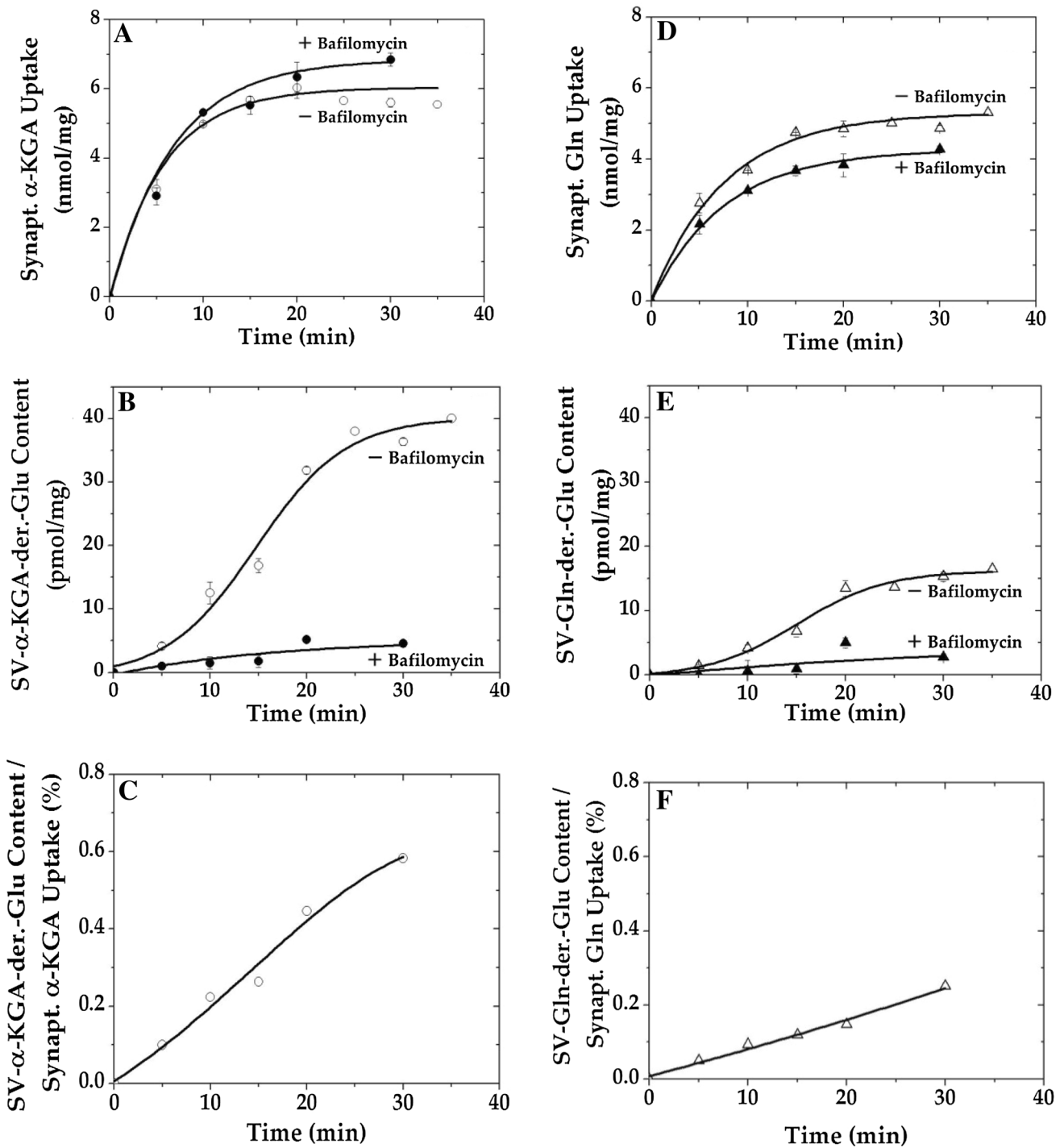
Fig. 4 Time course of accumulation of [^{14}C]α-KGA- and [^{3}H]glutamine-derived glutamate in synaptic vesicles within synaptosomes. Synaptosomes (256 µg of protein) were preincubated in the absence (*open circles*) or presence (*filled circles*) of 5 µM bafilomycin, and assayed for uptake of [^{14}C]α-KGA (**a**) and [^{3}H]glutamine (**d**) and for vesicular content of [^{14}C]α-KGA- and [^{3}H]glutamine-derived glutamate (**b, e**), as described in Fig. 3, with the following modifications. The incubation mixture contained 140 mM NaCl, 1.5 mM K$_2$HPO$_4$, 2 mM MgSO$_4$, 1.3 mM CaCl$_2$, 5 mM glucose 10 mM HEPES–NaOH (pH 7.4), and 53 µM [^{14}C]α-KGA *plus* 53 µM [^{3}H]glutamine (double labeling). The ratio of vesicular content/synaptosomal uptake (**c, f**) was calculated as described in Fig. 1

AAT. Our finding is consistent with the mass spectrometric detection of mitochondrial AAT peptide fragments in highly purified synaptic vesicles [45, 46].

There are only two genes known to encode for AAT, *GOT1* and *GOT2*, the former for cytosolic and the latter for mitochondrial AAT. Thus, vesicle-bound AAT is likely to
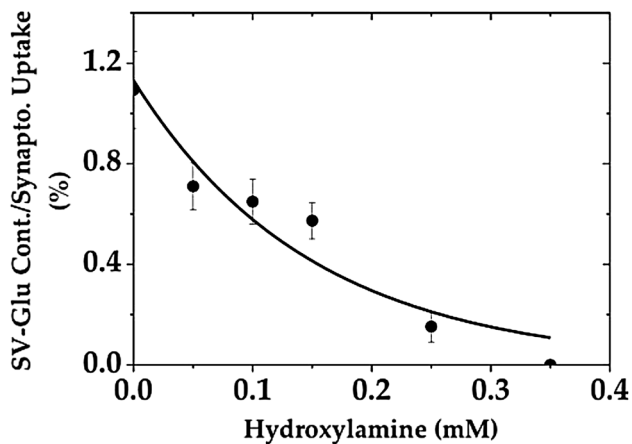
Fig. 5 Accumulation of α-KGA-derived glutamate in synaptic vesicles within synaptosomes is abolished by the AAT inhibitor hydroxylamine. Experimental conditions and procedures were essentially the same as described in Fig. 3, except that preincubation was carried out in the presence of various concentrations of hydroxylamine. The data represent the average with variation of two independent experiments

be encoded by *GOT2*, belonging to the mitochondrial type of AAT. Mitochondrial AAT is bound to the matrix side of the inner membrane of mitochondria [47–52]. In contrast, vesicular AAT is likely to be bound to the vesicle exterior, rather than its lumen side, since it is easily dissociated with salt (data not shown). It would function as a generator of the VGLUT substrate to be taken up into synaptic vesicles, whereas mitochondrial AAT would function as a generator of α-KGA to be transported out of mitochondria and/or to be metabolized via the TCA cycle.

Mitochondrial AAT is synthesized in a precursor form containing a 29-amino acid-presequence peptide, whereas cytosolic AAT lacks this peptide. The N-terminal 30-amino acid segment of the processed precursor is critical for recognition by the peptidase which removes the presequence peptide, forming the mature form of mitochondrial AAT [53]. Transport of the mitochondrial AAT precursor to mitochondria involves binding to hsp 70; the binding requires both the presequence element and the N-terminal region of mature form of AAT [54]. These factors are also required for import of the precursor protein into mitochondria [53]. The mechanism of transport and binding of the mitochondrial type of AAT to synaptic vesicles remains to be elucidated.

Physiological Significance

In order to maintain normal synaptic transmission, after synaptic vesicles release neurotransmitters, emptied vesicles require rapid transmitter refilling. The evidence that synaptic vesicles bear AAT producing glutamate readily available for VGLUT suggests an efficient mechanism for swiftly accumulating glutamate into vesicles. That is proposed to be through its local synthesis at the synaptic vesicle

site in the nerve ending, as opposed to the synthesis in the cytosol or mitochondria distant from synaptic vesicles. This is supported by the observation that vesicle-bound AAT has kinetic advantage over the cytosolic AAT described above. Having an aspartate K_m value substantially lower than its physiological concentrations [55, 56], vesicle-bound AAT would function at a reaction rate close to V_{max} in situ, whereas cytosolic AAT, with much lower aspartate affinity, would function at a rate far below V_{max}. Cytosolic AAT is likely to be functional at locations where aspartate concentration is high, such as those close to mitochondria, where it would play a role in the malate/aspartate shuttle. Cytosolic glutamate produced by this isoform would be transported into mitochondria in exchange for the efflux of aspartate from mitochondria, mediated by the aspartate/glutamate carrier [57–60].

Synaptosomes are capable of taking up α-KGA (Figs. 1a, 3a, 4a), as initially shown by Shank and Campbell [42, 61, 62]. We have provided evidence here that α-KGA taken up into synaptosomes results in vesicular storage of putative glutamate (Figs. 3c, 4b, 5c). This is compatible with the observation that exogenously supplied α-KGA produces releasable glutamate in a neuronal culture [63]. Shank and Campbell [42] have also shown that exogenous α-KGA is converted largely to glutamate in synaptosomes, suggesting a role for extraneuronally derived α-KGA as a precursor of the transmitter glutamate. One source of α-KGA in vivo could be astrocytes. Astrocytes in culture have been shown to release α-KGA [64, 65]. This release was increased when the culture was incubated in the presence of bicarbonate, suggesting that this α-KGA is largely produced through pyruvate carboxylation prominent in astrocytes, followed by the TCA cycle [64, 65]. These observations together raise the possibility that α-KGA supplied by astrocytes in vivo could serve in part as a precursor for synthesis of the vesicular pool of glutamate. Shank et al. [66] suggested that α-KGA derived from the oxaloacetate de novo synthesized by astrocyte-specific pyruvate carboxylase could serve as a precursor of the neurotransmitter pool of glutamate. Moreover, glutamate taken up into astrocytes has been shown to be converted substantially to α-KGA, as well as to glutamine [67–71]. Thus, the glutamate-glutamine cycle is not stoichiometric [41].

Another source of α-KGA would be nerve terminal mitochondria, where it is produced via the TCA cycle fueled by glucose and glutamine, the latter being supplied by astrocytes as well as from cerebrospinal fluid [47, 72–74]. Palaiologos et al. [75] have provided evidence consistent with the notion that α-KGA transported out of mitochondria, mediated by the α-KGA-malate exchanger [76–78], is utilized to synthesize releasable glutamate.

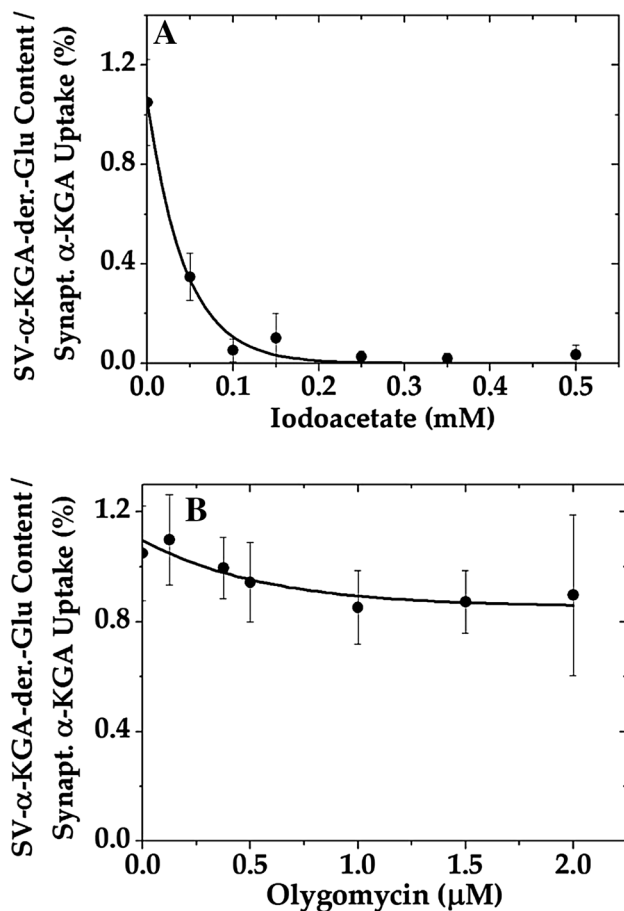Evidence presented here suggests that α-KGA, either exogenously supplied or endogenously produced from

Fig. 6 The glycolytic ATP synthase inhibitor iodoacetate, but not the mitochondrial ATP synthase inhibitor oligomycin, blocks vesicular accumulation of α-KGA-derived glutamate in synaptosomes. Experimental conditions and procedures were essentially the same as described in Fig. 3, except that preincubation was carried out in the presence of various concentrations of iodoacetate (**a**) or oligomycin (**b**). The data represent the mean±SD from three independent experiments

glutamine by glutaminase coupled with mitochondrial AAT and/or GDH, is converted to glutamate by extra-mitochondrial AAT in nerve terminals, and that glutamate, particularly the one re-synthesized by vesicle-bound AAT, is readily loaded into synaptic vesicles. This implies that vesicle-bound AAT has an important role in glutamate synaptic transmission. This notion is supported by a preliminary observation that injection of the AAT-selective inhibitor pyrazine-2,3-dicarboxylate [38] into the calyx of Held, a giant nerve terminal in the auditory brainstem, attenuated glutamate synaptic transmission (L. Y. Wang, personal communication). Cytosolic AAT could also contribute in part to production of glutamate to be taken up by synaptic vesicles. However, this mechanism would be less efficient because of its much lower affinity for aspartate, as described earlier. Nonetheless, the relative contribution of vesicle-bound and cytosolic AAT to the synthesis of the neurotransmitter pool

of glutamate remains to be determined, particularly in view of the immunocytochemical localization of cytosolic AAT in nerve terminals [79, 80].

We have previously shown that synaptic vesicles are endowed with the glycolytic ATP-generating enzymes glyeraldehyde-3-phosphate dehydrogenase/3-phospoglycerate kinase and pyruvate kinase [36, 37], and that ATP produced through glycolysis is preferentially harnessed to fuel vesicular loading of glutamate into synaptosomes [36]. Evidence is presented here that α-KGA-derived glutamate uptake into synaptic vesicles in synaptosomes is also fueled by glcolytically generated ATP. This is consistent with the notion of an efficient mechanism for rapid vesicular glutamate refilling [36, 81]; glycolytic ATP is produced prior to ATP synthesized in mitochondria.

The cellular model of memory long-term potentiation, observed under high frequency neuronal stimulation, involves modification of both pre- and post-synaptic elements [82, 83]. Evidence indicates that neuronal activity presynaptically regulates neurotransmitter quantal size under physiological conditions [84]. Wilson et al. [85] have demonstrated that chronic increase in neuronal activity regulates VGLUT expression and the amount of glutamate released per vesicle. We have observed that the *process* of vesicular accumulation of α-KGA-derived and glutamine-derived glutamate in synaptosomes is enhanced by depolarization, which however suppresses synaptosomal uptake. This is relevant to activity-dependent alteration in the presynaptic nerve terminal. Depolarization is known to induce reversal of Na^+-dependent plasma membrane transport [86]. The enhancing effect of depolarization on glutamate accumulation in synaptic vesicles could reflect a presynaptic acute homeostatic response in order to sustain glutamate transmission. These results are consistent with our previous observation that pre-exposure of synaptosomes to depolarization conditions leads to augmented vesicular glutamate content [87]. The mechanism underlying this acute depolarization-induced alteration in the synaptic vesicular content of α-KGA/glutamine-derived glutamate remains to be elucidated.

Vesicular Accumulation of Glutamine-Derived Glutamate vs. α-KGA-Derived Glutamate

Bradford et al. [72] have previously shown that glutamine is taken up into synaptosomes, where it is transformed to releasable glutamate by glutaminase. Our studies suggest that glutamine taken up into synaptosomes is incorporated into synaptic vesicles largely as glutamate. This occurs despite the fact that synaptic vesicles are essentially free of glutaminase [38, 45, 46]. Five pathways are possible for this occurrence: (a) Glutamate produced by mitochondrial glutaminase [88] is transferred to the cytoplasm and taken up
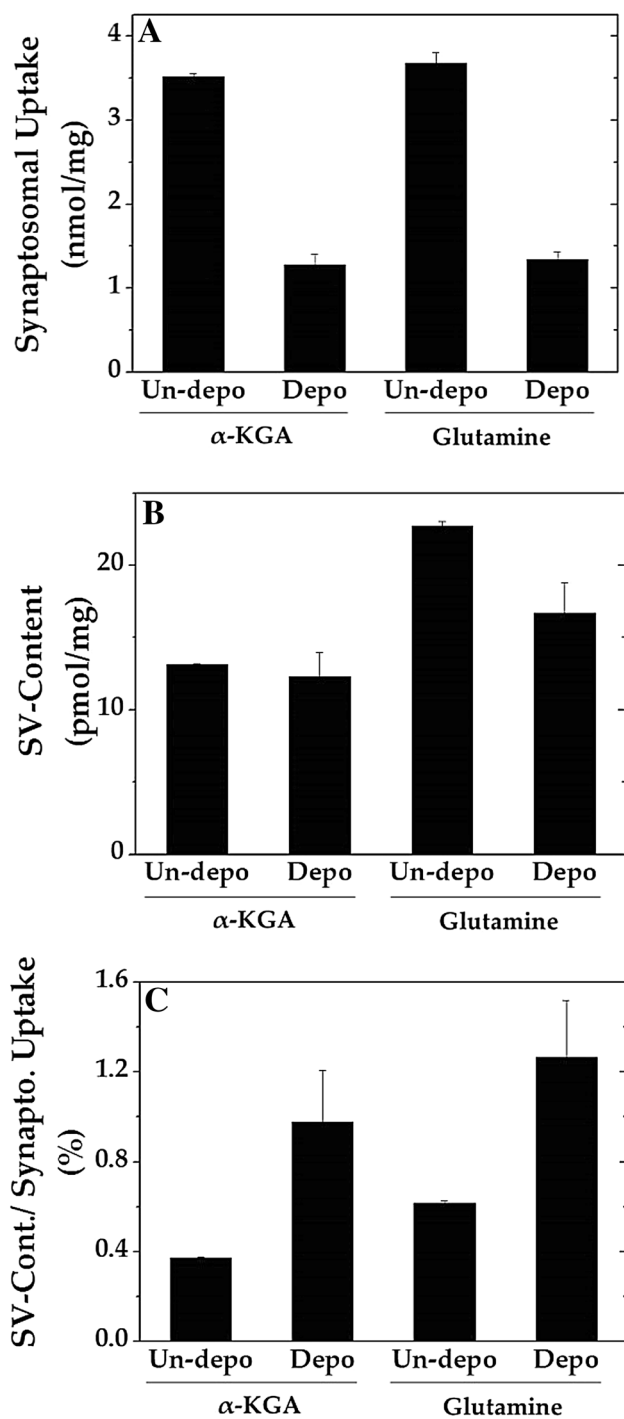
Fig. 7 Depolarization facilitates vesicular accumulation of α-KGA- and glutamine-derived glutamate. Depolarization enhances vesicular α-KGA- or glutamine-derived glutamate content in synaptosomes. Synaptosomes (P2 fraction: 374–387 μg) from fresh rat cerebrum were incubated at 37 °C for 30 min with 53 μM [^{14}C]α-KGA *plus* [^{3}H]glutamine (double labeling), each under depolarizing (with 56 mM K$^+$ replacing 56 mM NaCl) or the non-depolarizing conditions described in Fig. 3, and bafilomycin-sensitive vesicular glutamate content (**b**) as well as synaptosomal uptake (**a**) determined. The ratio of vesicular content/synaptosomal uptake (**c**) was calculated as described in Fig. 1. The data represent the average with variation from two independent experiments

by synaptic vesicles. (b) Glutaminase-generated glutamate is first transaminated by AAT in mitochondria, producing α-KGA and aspartate, both of which are then transported out of mitochondria and utilized by extra-mitochondrial AAT, reforming glutamate and oxaloacetate. Glutamate thus re-synthesized is taken up into synaptic vesicles. (c) Glutamine-derived glutamate is converted to α-KGA in the mitochondria matrix through oxidative deamination mediated by GDH. α-KGA thus formed is then transported out of mitochondria and transformed by extra-mitochondrial AAT to glutamate, which is then taken up into synaptic vesicles. (d) Combination of (b) and (c). (e) Combination of (a), (b) and/or (c).

Pathway *a* is unlikely since mitochondrial glutaminase is localized to the mitochondrial inner membrane [89] and its matrix side [48, 49, 52]; this implies that the glutamine to glutamate conversion occurs in the matrix. Palaiologos et al. [75] have provided evidence, using cultured cerebellar granule neurons, suggesting that AAT activity and α-KGA efflux from mitochondria are required for re-synthesis of releasable glutamate from glutamine. Yudkoff et al. [43] have demonstrated that reformation of glutamate from glutamine-derived α-KGA, as well as the malate/aspartate shuttle, is rapid in synaptosomes. Synaptosomal mitochondria have high AAT activity [47, 74, 90], which would produce substantial amounts of α-KGA and aspartate from glutamine-derived glutamate for export to the cytoplasm, supplying the substrates for extra-mitochondrial AAT. These lines of evidence support pathway *b*. On the other hand, Tildon et al. [91] and McKenna et al. [92] have provided evidence that in synaptosomes GDH plays a major role in converting glutamine-derived glutamate to α-KGA for the TCA cycle. GDH activity is significantly higher in synaptic mitochondria than in non-synaptic mitochondria, in particular the heavier synaptic mitochondrial fraction [47, 74, 90, 93]. These observations support pathway *c*. Moreover, over-expression of GDH has recently been shown to cause increased synaptic release of glutamate [94]. Overall pathway *d* would be the more likely possibility. Pathway *b* generates not only α-KGA but also aspartate, which is not directly produced by pathway *c*. α-KGA generated by pathway *c* could be transported out of mitochondria and/or enter the TCA cycle, in parallel leading to replenishment of the oxaloacetate which had exited from the TCA cycle as a result of its conversion to aspartate. The relative contribution of these pathways remains to be investigated.

Bradford et al. [72] and Hamberger et al. [73] have provided biochemical evidence that glutamine serves as the major precursor for synthesis of the neurotransmitter glutamate. This, together with the localization of glutamine synthetase to astrocytes [95] and other evidence [55, 96, 97], has led to the concept that the glutamate-glutamine cycle plays a central role in synthesis of the neurotransmitter pool of
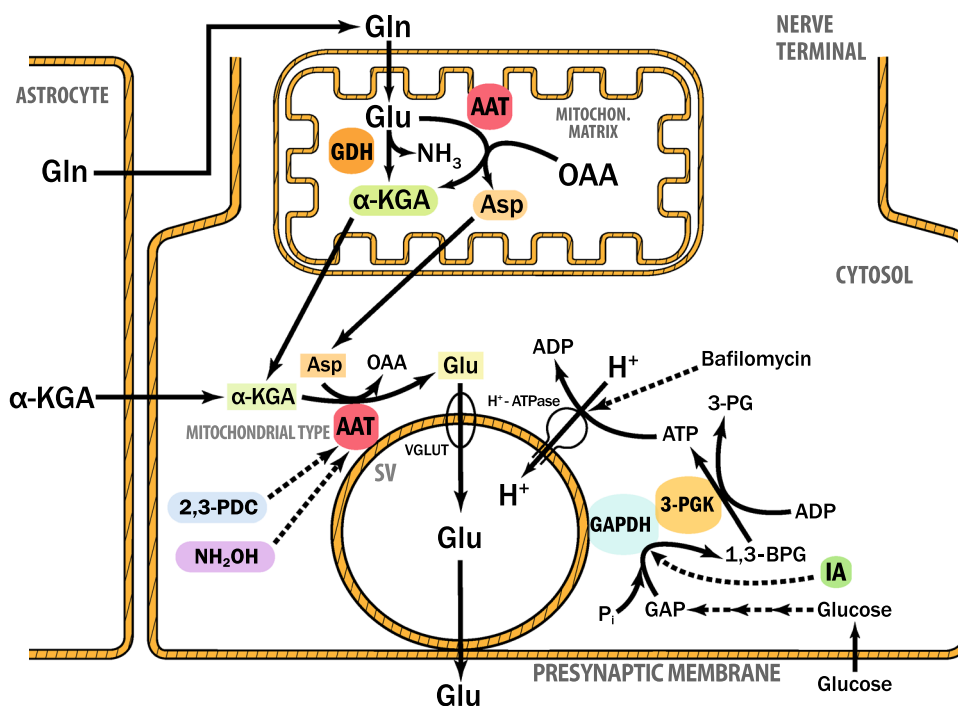
Fig. 8 Model for synthesis and loading of neurotransmitter glutamate. Proposed is an efficient mechanism for synthesis of the VGLUT substrate glutamate for immediate uptake into synaptic vesicles. The VGLUT substrate is locally produced by vesicle-bound AAT from α-KGA and aspartate and readily transported into synaptic vesicles, fueled by vesicle-bound glycolytic ATP-generating enzymes. α-KGA derives from nerve terminal mitochondria, where it is generated (1) thorough the TCA cycle, (2) by AAT and (3) by GDH from glutamate, which is produced by glutaminase from astrocyte-derived glutamine. α-KGA is also supplied by adjacent astrocytes. Aspartate derives from mitochondria, where it is produced by AAT from oxaloacetate with glutamate as the amino donor. *AAT* aspartate aminotransferase, *α-KGA* α-ketoglutarate, *Asp* aspartate, *1,3-BPG* 1,3-bisphosphoglycerate, *GAP* glyceraldehyde-3-phophate, *GAPDH* glyceraldehyde-3-phophate dehydrogenase, *GDH* glutamate dehydrogenase, *Glu* glutamate, *Gln* glutamine, *IA* iodoacetate, *OAA* oxaloacetate, *P$_i$* inorganic phosphate, *2,3-PDC* 2,3-pirazinedicarboxylate, *3-PG* 3-phophoglycerate, *3-PGK* 3-phosphoglycerate kinase, *SV* synaptic vesicle, *VGLUT* vesicular glutamate transporter, *v-H$^+$-ATPase* v-type proton-pump ATPase

glutamate [65, 98, 99]. According to this model, glutamate released from the nerve terminal is taken up by adjacent astrocytes, where it is converted to glutamine by glutamine synthetase, which is highly enriched in astrocytes. Glutamine thus produced is released from astrocytes and taken up by adjacent nerve terminals, where it is hydrolyzed to glutamate by mitochondrial glutaminase. Glutamate thus produced provides the neurotransmitter pool of glutamate and is released. Although this model is well recognized, and the glutamine-glutamate conversion is essential for maintaining activity-dependent release of glutamate and the function of active synapses, the role of the glutamate-glutamine cycle in glutamate synaptic transmission, in particular spontaneous glutamate release and baseline glutamatergic synaptic transmission is not entirely clear [84, 100]. Kam and Nicoll [100] and Masson et al. [101] have provided evidence that spontaneous glutamate transmission occurs even in the absence of glia cells or of an exogenous supply of glutamine, as well as in the absence of mitochondrial glutaminase. This raises the possibility that glutamate synthesized from part of the α-KGA derived from glucose via the neuronal TCA cycle

could be responsible for spontaneous glutamate transmission. This α-KGA is constantly produced in nerve terminals, independent of astrocyte metabolism or synaptically released glutamate, a major source of glutamine synthesis in astrocytes. Alternative carbon sources such as other amino acids, fatty acids, and ketone bodies, however, cannot be ruled out [41, 71].

Palaiologos et al. [75] have provided evidence suggesting that the conversion of glutamine-derived glutamate to α-KGA by mitochondrial AAT, as well as the efflux of α-KGA and aspartate from mitochondria, is essential for re-synthesis of releasable glutamate in the extra-mitochondrial compartment. This notion is consistent with our observations presented here. We propose that α-KGA is the principal *immediate* precursor for synthesis of the neurotransmitter pool of glutamate, regardless of whether α-KGA is derived from glutamine (through the actions of glutaminase and AAT/GDH) or from glucose via the TCA cycle, or of whether it is directly supplied from astrocytes (Fig. 8). Vesicle-bound AAT-coupled glutamate biosynthesis from α-KGA, together with local synthesis of ATP by

vesicle-bound GAPDH/3-phosphoglycerate kinase, could represent an effective mechanism for rapid refilling of synaptic vesicles with glutamate to sustain normal synaptic transmission.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest regarding the work reported here.

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