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ORIGINAL PAPER

Glutamate Release

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Abstract Our aim was to review the processes of glutamate release from both biochemical and neurophysiological points of view. A large body of evidence now indicates that glutamate is specifically accumulated into synaptic vesicles, which provides strong support for the concept that glutamate is released from synaptic vesicles and is the major excitatory neurotransmitter. Evidence suggests the notion that synaptic vesicles, in order to sustain the neurotransmitter pool of glutamate, are endowed with an efficient mechanism for vesicular filling of glutamate. Glutamate-loaded vesicles undergo removal of Synapsin I by CaM kinase II-mediated phosphorylation, transforming to the release-ready pool. Vesicle docking to and fusion with the presynaptic plasma membrane are thought to be mediated by the SNARE complex. The Ca^{2+}-dependent step in exocytosis is proposed to be mediated by synaptotagmin.

Keywords VGLUT · Excitatory synapse · Glycolytic ATP · Aspartate aminotransferase · Local synthesis · Synapsin I · Synatotagmin · SNARE

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Abbreviations

AAT	Aspartate aminotransferase
CNS	Central nervous system
CaM-PK	Calmodulin-dependent protein kinase
GABA	γ-Aminobutyric acid
mepp	Miniature end-plate potential
RIM	Rab3 interacting molecule
RIM-BP	RIM-binding protein
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
TCA	Tricarboxylic acid
VGLUT	Vesicular glutamate transporter

Synaptic Transmissions and Quantal Theory

Our review focuses on release of glutamate from presynaptic terminals in the vertebrate CNS, in crustacean neuromuscular junctions, and the squid giant synapse. Biochemical, structural and functional properties are considered. A summary of the development of the synaptic concept provides a technical background and historical perspective.

Early Morphological, Pharmacological, and Physiological Evidence for Synapses

Ramon y Cajal [1] used Golgi's silver staining technique to visualize microscopically the entire structure of individual neurons. Cajal described such structures as the dendritic tree and traced the course of axons to their contacts on target neurons. Many of his drawings used arrows to illustrate how neurons integrated synaptic information (e.g.: Parallel fiber and climbing fiber inputs to Purkinje cell, Fig. 104, p 121).

Sherrington [2] furthered the concept of neuron theory by suggesting that the direction of impulse traffic was regulated at the neuronal contact for which he coined the term "SYNAPSE" from the Greek word meaning to clasp. The implication was that there was a functional contact between nerve cells for the transmission of nerve impulses from one neuron to another.

Loewi [3] demonstrated that acetylcholine was released from the vagus nerve innervating the heart. However, the possibility that chemical neurotransmission occurred in the brain was rejected early in favor of electrotonic transmission primarily because of the apparent short synaptic latency. Eccles [4] resolved this issue in favor of chemical neurotransmission. A schematic summary of presynaptic and postsynaptic processes characteristic of all chemical synapses is provided in Fig. 1.

Physiological Evidence for Packaged Neurotransmitter

Katz et al. [5, 6], using recently developed microelectrodes [7], penetrated frog muscle fibers and recorded millivolt signals in the region of motor nerve terminals (end-plates). These small, spontaneously occurring potentials were named miniature end-plate potentials (mepp's) because they were similar in shape to the end-plate potentials (epp's) evoked by action potentials in the presynaptic nerve. Both epp's and mepp's decreased in amplitude as the recording microelectrode was placed further from the end-plate region.

Their amplitude also decreased following application of curare to block the postsynaptic receptors and conversely increased by applying blockers of acetylcholine esterase. Moreover, extracellular calcium ion (Ca^{2+}) concentration (below 2 mM) decreased the amplitude of the epp's but not the mepp's. Similarly, repetitive presynaptic nerve stimulation resulted in a decrease in the amplitude of epp's but not mepp's. These observations led to the concept that the presynaptic action potential evoked a postsynaptic response that was made up of hundreds of smaller events which Katz called quanta. The quantal size was not reduced by most treatments. However, it was later shown that hemicholinium-3 did reduce the amplitude of mepp's but only after repetitive simulation [8]. This was explained as a decrease in amount of acetylcholine stored per quanta following the block of choline uptake into the presynaptic nerve terminal, which was necessary for acetylcholine synthesis.

Quantal release has also been detected as stepwise changes in capacitance [9] and small current changes measured with etched carbon-fiber microelectrodes [10]. Combining these techniques has allowed the detection of incomplete vesicular fusion known as kiss-and-run exocytosis (for review, see Ref. [11]).

Electron Microscopy of Presynaptic Vesicles

About the time Katz recorded mepp's, anatomists using the electron microscope found vesicles in presynaptic terminals in the CNS [12–14]. This led to the concept that the "quanta" were the result of a fixed amount of neurotransmitter stored in "presynaptic vesicles". The quantal theory of Katz stated that vesicles are the storage site of transmitter that was released either spontaneously or by presynaptic action potentials. The released neurotransmitter crossed a synaptic cleft before activating their specific receptors in the postsynaptic membrane.

The Isolation of Presynaptic Vesicles

Whittaker [15] isolated presynaptic vesicles from the mammalian brain by high speed ultracentrifugation. Highly purified presynaptic vesicles were shown to accumulate the neurotransmitter glutamic acid in an energy dependent manner [16].

Quantal Release at Glutamatergic Synapses

The general importance of quantal release was questioned and examined at the glutamatergic synapses of the crayfish neuromuscular junction [17, 18], in the frog spinal cord [19], and of the squid stellar ganglion [20].

Excitatory Actions of Acidic Amino Acids

The majority of excitatory synapses in the brain are not mediated by acetylcholine, serotonin, or norepinephrine. A large number of acidic amino acids have been electrophoretically injected onto neurons in the brain and have produced strong excitation [21–24]. There is a similarity between the depolarizing action produced by amino acids and the depolarizing action of excitatory synapse. An example is shown in the frog cerebellum where glutamate was compared with EPSPs of climbing and parallel fibers [25].

Difficulty in Acceptance of Glutamate as a Neurotransmitter

Despite the pharmacological evidence suggesting a neurotransmitter role for glutamate, many were initially skeptical that glutamate could function as a natural neurotransmitter. There were three major reasons, the first teleological. Glutamate occurs as a major biochemical, not

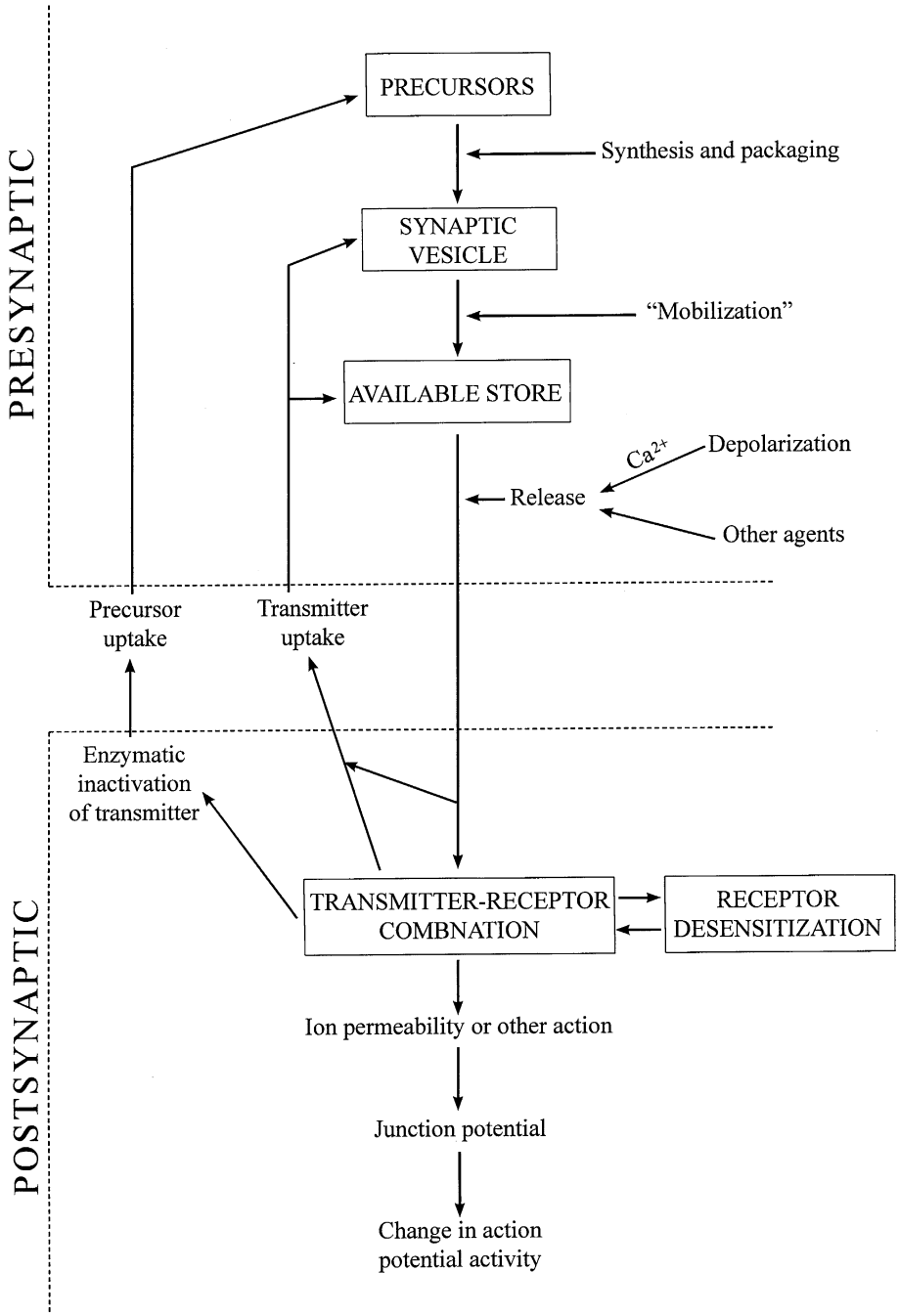

Fig. 1 Diagram of synaptic transmission

just in neurons, but in all cells in the entire biological system. As such, glutamate is involved in a number of other important cellular processes, such as energy and nitrogen metabolism and the synthesis of proteins, peptides, nucleic acids, and folic acid, as well as the inhibitory neurotransmitter GABA. In contrast, all the other well recognized neurotransmitters (acetylcholine, GABA, norepinephrine, dopamine, and serotonin) are synthesized only in neurons and some endocrine cells, and function solely as neurotransmitters or hormones.

Second, although Curtis et al. [21] clearly demonstrated rapid, reversible, and potent effects of glutamate in evoking neuronal firing in the majority of CNS neurons, they concluded, based upon its apparent non-specific action, that glutamate does not serve as a neurotransmitter. It had been thought that neurotransmitters act only on specific neurons, based upon similar observations with all other classical neurotransmitters.

Third, evidence was lacking at the time for accumulation of glutamate in synaptic vesicles. Studies by Mangan

and Whittaker [26], Rassin [27], and Kontro et al. [28] showed that glutamate was not enriched in isolated synaptic vesicles. Likewise, there was no evidence that synaptic vesicles are capable of concentrating glutamate [29]. Based on this and other evidence, it was proposed that glutamate is released from the cytosol, rather than from the synaptic vesicle [30]. This failed to provide support for the notion that glutamate functions as a neurotransmitter; well-established neurotransmitters had been found concentrated in synaptic vesicles.

Release of Glutamate

Ca^{2+} -dependent release is an important criterion for a substance to be considered a candidate for neurotransmitter. De Belleroche and Bradford [31] showed that high K^+- or veratrine-induced depolarization of synaptosomes (from cerebral cortex) caused release of glutamate. Sandoval et al. [32] provided evidence for Ca^{2+} dependent, high K^+-induced release of glutamate from hippocampal synaptosomes. Hamberger et al. [33] also provided evidence for Ca^{2+}-dependent glutamate release using a hippocampus slice preparation. However, in all cases it was concluded that the release originated from the cytosol. Subsequently, Nicholls and Sihra [34] and Tibbs et al. [35] clearly demonstrated that depolarization-induced endogenous glutamate release from synaptosomes (from cerebral cortex) occurs rapidly in a Ca^{2+}-dependent manner, originating most likely from synaptic vesicles (for review, see Ref. [36]).

Evidence for Accumulation of Glutamate in Synaptic Vesicles

Critical for synaptic transmission is the uptake of glutamate into synaptic vesicles. Clear biochemical demonstration of vesicular neurotransmitter loading requires preparation of synaptic vesicles free of contamination with other intracellular organelles and plasma membranes. Naito and Ueda [16] isolated synaptic vesicles using antibodies specific to Synapsin I [37], a synaptic vesicle-specific protein [38–41]. These vesicles were free from other organelles and plasma membranes, and demonstrated to take up glutamate in an ATP-dependent manner. ATP-dependent glutamate uptake into synaptic vesicles was shown to be driven by an electrochemical proton gradient, specific to glutamate and markedly stimulated by low millimollar Cl^- [42]. Disbrow et al. [43] also reported ATP-dependent glutamate uptake into crude and partially purified synaptic vesicles. However, the ATP stimulation was marginal. This is most likely the result of two factors: Cl concentration (see below) and organelle and other sealed membrane contaminants. Moreover, specificity to glutamate was not addressed. Neither ATP nor low millimolar Cl^- was included in the incubation medium by previous investigators [29]. The presence of low millimolar Cl^- as well as ATP in the incubation medium, not to mention the use of a highly purified synaptic vesicle preparation, proved critical for clear demonstration of glutamate accumulation into synaptic vesicles [16, 42].

Storm-Mathisen et al. [44] provided immunocytochemical evidence for glutamate accumulation in synaptic vesicles, distinct from GABA containing synaptic vesicles. Maycox et al. [45] also showed that highly purified synaptic vesicles by chromatography on controlled-pore glass beads take up glutamate at the expense of an electrochemical gradient. Moreover, Burger et al. [46] demonstrated that glutamate is concentrated in rapidly isolated synaptic vesicles. Of interest, glutamate accumulated into synaptic vesicles was not well maintained in the absence of an electrical proton gradient [47]. This could explain, at least in part, the initial failure to demonstrate enrichment of glutamate in isolated synaptic vesicles [26–28]. These lines of evidence together provided strong support for the concept that glutamate has a neurotransmitter function.

ATP energy is harnessed by vesicle-bound proton-pump ATPase to generate an electrochemical proton gradient, which provides the driving force for the vesicular glutamate transporter [16, 42, 45, 48–65]. This vesicular uptake system is not only specific to glutamate, but also unique in sensitivity to Cl^- [42]. Thus, the vesicular glutamate transporter, VGLUT, does not recognize aspartate [42, 49, 50, 52, 54–60, 63, 66–69]. Of particular interest, the vesicular glutamate uptake system is stimulated by low concentrations and inhibited by high concentrations of Cl^-.

This unique property was initially observed by Naito and Ueda [42], and is now well established [48–50, 52–54, 56–58, 60, 62–66, 70, 71]. Moreover, affinity for glutamate is low [42, 49, 50, 52–54, 56–60, 63]. At physiologically relevant Cl^- concentrations, both the membrane potential and the pH gradient contribute to the driving force [42, 51, 53, 56, 64]. These properties render VGLUT distinct from the plasma membrane glutamate transporter [72]. The latter utilizes the sodium gradient as the driving force, does not distinguish between glutamate and aspartate, is not subject to bimodal chloride modulation, and has high affinity for glutamate [73, 74]. VGLUT's low affinity for glutamate is compatible with high cytosolic concentrations of glutamate, whereas the plasma membrane transporter requires high affinity for glutamate, since the extracellular concentration of glutamate is maintained at very low levels. These observations led to the model that the vesicular glutamate uptake system consists of proton-pump ATPase and a vesicular glutamate transporter [54, 65, 72, 75–77].

Mechanism of Bimodal Modulation by Cl^- of Vesicular Glutamate Transport

Low millimolar Cl^- concentrations increase V_{max} with little change in K_m for glutamate, whereas further increase in Cl^- concentrations reduces V_{max} and increases K_m for glutamate [42, 53]. The inhibitory effect of high Cl^- concentrations is attenuated by high glutamate concentrations [42, 53, 64]. The Cl^- stimulation was ascribed to an optimal pH (around 6.8) created in the vesicle lumen, due to low influx of Cl^- [51]. This pH may be optimal for activating VGLUT, possibly by facilitating release of translocated yet bound glutamate. Hartinger and Jahn [78] and Juge et al. [63, 70] have provided evidence suggesting that that the Cl^- stimulation occurs due to activation of VGLUT by binding to an external regulatory site.

On the other hand, Schenk et al. [64] have provided evidence suggesting that VGLUT1 has membrane potential-driven Cl^- transport function, and causes glutamate flux by a membrane potential-dependent Cl^-/glutamate antiporter mechanism, ruling out the possibility that Cl^- binds to a regulatory site of VGLUT1. Chloride conductance by VGLUT1 had also been shown by Bellocchio et al. [56]. VGLUT1 was also proposed to act as a proton/glutamate antiporter, which is thought to be the sole mechanism when Cl^- is absent in the vesicle lumen [64]. The proton/glutamate antiporter model for vesicular glutamate transport was also previously proposed [79].

Thus, in the presence of Cl^- in the vesicle lumen, membrane potential alone can transport glutamate into synaptic vesicles, whereas in its absence, glutamate transport does not occur even in the presence of membrane potential. When a low concentration of Cl^- is present in the extravesicular medium, a Cl^- influx-induced pH gradient is formed, which allows glutamate influx. The contribution of the pH gradient is greater when Cl^- is absent in the lumen [64]. In the absence of intravesicular Cl^-, the pH gradient dependency of glutamate uptake is higher when extravesicular glutamate concentration is lower, suggesting that glutamate and Cl^- compete for transport into vesicles. This was also suggested by Bellocchio et al. [56]. Thus, higher Cl^- concentration inhibits glutamate uptake into vesicles. This explanation for high Cl^--induced, lowered vesicular glutamate uptake is in agreement with the notion that Cl^- interacts with the glutamate binding site of VGLUT [42].

In contrast, Juge et al. [63] have ascribed the high Cl^--induced inhibition of vesicular glutamate uptake to collapse of the driving force membrane potential, due to an increase in pH gradient. However, under conditions in which the membrane potential was enhanced by nigericin (K^+/H^+ exchanger) or A23187 (Ca^{2+} or Mg^{2+}/H^+ exchanger) and maintained constantly high in the presence of various concentrations of Cl^-, Cl^--dependent suppression of vesicular glutamate was still observed [51, 53]. In agreement with observations by Hartinger et al. [78], studies by Juge et al. [63, 70] suggest that low Cl^- activates VGLUT2 by binding to an allosteric site. VGLUT2 did not cause Cl^- transport into liposomes devoid of proton-pump ATPase when the membrane potential was generated by valinomycin.

However, when proton-pump ATPase is present in liposomes, low Cl^--stimulated glutamate uptake (as well as high Cl^--suppressed glutamate uptake) via VGLUT1 is substantially reduced by dissipating the pH gradient [64]. Thus, the mechanism underlying the Cl^- bimodal modulation remains controversial. Mention may be made, however, that intact synaptic vesicles are endowed with proton-pump ATPase. Hence, although the membrane potential alone can cause glutamate transport into vesicles, membrane potential-driven Cl^- influx would also occur under physiological conditions, resulting in formation of a pH gradient which could, in addition, significantly contribute to the driving force [42, 51, 53, 56, 64].

Under physiological conditions, synaptic vesicles would contain some Cl^- in the lumen and be surrounded by a low millimolar cytosolic concentration of Cl^-. In this situation, according to the model proposed by Schenk et al. [64], glutamate transport into synaptic vesicles would occur by both the membrane potential-dependent Cl^-/glutamate exchange and proton/glutamate symport mechanisms. The membrane potential is thought to play a role in changing the conformation of VGLUT. This is supported by observations suggesting that the membrane potential alters the affinity of VGLUT for glutamate; the K_m value for glutamate is increased by dissipating the membrane potential [18, Lee and Ueda, unpublished data] and decreased by increasing the membrane potential [53] in the absence or presence of low millimolar Cl^-. When synaptic vesicles are exposed to non-physiological, high concentrations of Cl^-, vesicular glutamate uptake is reduced. This could be partly due to reduced Cl^- efflux, resulting in reduced glutamate influx, and partly due to competition between glutamate and Cl^- for the vesicular entry site of VGLUT [42, 53, 54, 64]. Evidence indicates that glutamate uptake V_{max} is reduced by high concentrations of Cl^-, compared to that at low millimolar Cl^-, whereas K_m for glutamate is increased [42, 53].

Recently, Preobraschenski et al. [80] presented evidence that Cl^- translocation is an intrinsic property of VGLUT, but not coupled to glutamate transport. Thus, high Cl^- concentrations can reduce vesicular glutamate by competing for the glutamate transport site. The former property is consistent with observations by Bellocchio et al. [56] and Schenk et al. [64], but the latter contradicts the notion that

VGLUT mediates Cl^-/glutamate exchange [53, 64]. It is proposed that the stimulatory effect of low millimolar Cl^- is due to VGLUT activation by binding to an allosteric site in agreement with Hartinger et al. [78] and Juge et al. [63, 70]. The apparent discrepancy between the observations of Schenk et al. [64] and Preobraschenski et al. [80] and those of Juge et al. [70], with respect to the ability of VGLUT to mediate Cl^- flux, could be due to the difference in lipid composition of the proteoliposomes used. Liposomes used by Schenk et al. [64] and Preobraschenski et al. [80] consisted of phospholipid mixtures, whereas those used by Juge et al. [70] contained basically a single phospholipid component, phosphatidylcholine. It is feasible that phospholipid composition could affect transport function. Glutamate uptake activity in proteoliposomes composed of a phospholipid mixture [64] was much higher than in proteoliposomes comprised of a phosphatidylcholine only [70]. Carlson et al. [26] had also shown that a phospholipid mixture, not a single phospholipid, is required for optimal ATP-dependent glutamate uptake activity in a reconstituted system.

Vesicular Glutamate Transporter

The vesicular glutamate transporter protein had eluded identification for some time. In 2000, Takamori et al. [55] and Bellocchio et al. [56] demonstrated that a brain specific-isoform of the sodium-dependent inorganic phosphate transporter [81] functions as VGLUT. This was soon followed by identification of two additional isoforms, VGLUT2 [57–60, 67, 68, 82] and VGLUT3 [61, 62, 83, 84]. These isoforms differ largely in N- and C-terminal domains, but exhibit similar uptake characteristics [57–60, 62, 67, 83, 84]. They belong to the SLC 17 family [65]. In general, they have complementary distributions in the adult brain. However, in some glutamatergic nerve terminals and certain endocrine cells, as well as at early developmental stages, VGLUT1 and VGLUT2 are colocalized [85–92, 96]. VGLUT1 and VGLUT2 expression levels have been demonstrated to control quantal size [93–95]. VGLUT3 is peculiar in that it is expressed in dendrites, astrocytes and non-nervous tissues as well as in nerve terminals [84, 86, 96–98]. These observations suggest their involvement in different synaptic functions. VGLUT1 has been suggested to have a role in modulating transmitter release probability and synaptic plasticity, as well as in the reserved pool of vesicles [57, 60, 86, 87, 99, 100]. Colocalization of VGLUT 3 and vesicular acetylcholine and monoamine transporters has been implicated in motor control, reward behavior, and neuropsychiatric disease [84]. Subsequent studies indicate that all VGLUTs are colocalized with subsets of vesicular GABA and acetylcholine transporters. VGLUT2 is present in additional subsets of vesicles containing dopamine and norepinephrine, and VGLUT3 in an additional subset of serotonin-containing vesicles (for review, see Ref. [101]). Thus, VGLUT expression levels can modulate the vesicle's capacity to accumulate these transmitters. This co-phenotype has behavioral implications.

Energy Source Required for Vesicular Glutamate Uptake

Synaptic transmission depends on glycolysis [102], which generates the smaller pool of ATP. Global cellular ATP levels are hardly changed under those hypoglycemic conditions which cause initial abnormal synaptic transmission [103, 104]. Substitution with pyruvate, the final product of glycolysis, which feeds into mitochondria, producing the majority of ATP, does not restore normal synaptic transmission [102], even though tissue levels of ATP return to normal [103]. Fleck et al. [105] showed that hypoglycemic conditions which reduce glutamate release did not significantly alter cellular ATP content. These observations raised the possibility that a glycolytic intermediate or a minor pool of ATP locally synthesized might play a critical role in glutamate release. Ikemoto et al. [106] have provided evidence suggesting that glycolytically generated ATP, rather than mitochondrial ATP, plays a major role in providing energy for vesicular glutamate accumulation. The glyceroaldehyde-3-phosphate dehydrogenase (GAPDH) inhibitor iodoacetate suppressed glutamate accumulation into vesicles, whereas the mitochondrial ATP-synthetase inhibitor oligomycin had minimal effects. These observations are compatible with the finding by Shepherd and Harris [107] that approximately 50 % of hippocampal CA3/CA1 nerve terminals are devoid of mitochondria.

Of importance, synaptic vesicles were found to be endowed with the glycolytic ATP-generating enzyme complex, GAPDH/3-phosphoglycerate kinase, which is harnessed to generate energy for immediate active transport of glutamate into synaptic vesicles [106], consistent with proteomic studies [108–110]. Evidence suggests that a sub-population of synaptic vesicles bear pyruvate kinase, which is also capable of supporting vesicular glutamate uptake [111]. These observations together support the notion that locally synthesized glycolytic ATP is the major energy source harnessed for efficiently refilling of synaptic vesicles with glutamate [112]. It is interesting to note that the vital role of local synthesis of glycolytic ATP in fast axonal transport of synaptic vesicles has also been demonstrated recently [113].

Precursor for VGLUT Substrate

In eukaryotes, glutamate is synthesized by three enzymes:

(a) amino acid aminotransferase

$$\alpha\text{ - Ketoglutarate} + \text{Aminoacid} \rightleftharpoons \text{Glutamate} + \alpha\text{ - Ketoacid}$$

(b) glutamate dehydrogenase

$$\alpha\text{ - Ketoglutarate} + NH_4^+ + \underset{(NADPH)}{NADH} + H^+ \rightleftharpoons \text{Glutamate} + \underset{(NADP^+)}{NAD^+} + H_2O$$

(c) glutaminase

$$\text{Glutamine} + H_2O \rightarrow \text{Glutamate} + NH_3$$

Which enzyme or enzymes are responsible for formation of the neurotransmitter pool of glutamate remains to be fully elucidated. Glutamine is widely thought to be the principal precursor for synthesis of the neurotransmitter glutamate. Bradford et al. [114] and Hamberger et al. [33], using brain synaptosomes and hippocampal slices, respectively, incubated with radioactive glutamine and glucose, provided biochemical evidence that glutamine is the major substrate for the neurotransmitter pool of glutamate. This, together with specific localization of glutamine synthetase in astrocytes [115] and glutaminase enrichment in synaptosomes, particularly in mitochondria therein [116], as well as the notion of the glutamate–glutamine cycle [117, 118], has led to the concept that the glutamate–glutamine cycle plays a central role in supplying a precursor for synthesis of the neurotransmitter glutamate [119–121]. According to this model, released glutamate from nerve terminals is largely taken up by astrocytes, where it is converted to glutamine, which is then transported out to the extracellular space. The released glutamine is taken up by neurons and converted back to glutamate by glutaminase in mitochondria. Glutamate thus produced and released from mitochondria had been thought to be utilized as the neurotransmitter glutamate.

In support of this notion is evidence that phosphate-activated glutaminase is localized to the exterior of the inner mitochondrial membrane [122, 123]. However, other evidence indicates that the active site of this enzyme is on the matrix side of the mitochondrial inner membrane [124–128]. Indeed Palaiologos et al. [129] provided evidence suggesting that glutamate produced from glutamine is transformed to α-ketoglutarate by aspartate aminotransferase (AAT) in mitochondria [130] and exported to the cytoplasm, mediated by the dicarboxylic acid exchanger [131, 132]. α-Ketoglutarate released from mitochondria was then converted to glutamate, which is released in a Ca^{2+}-dependent manner. Glutamate was assumed to be resynthesized by AAT in the cytosol [129, 133].

Takeda et al. [134] have demonstrated that isolated synaptic vesicles are capable of synthesizing glutamate from α-ketoglutarate and L-aspartate (as the specific amino donor) by vesicle-bound AAT for immediate uptake. Glutamine was not an effective substrate for glutamate synthesis, consistent with the vesicle's deficiency in glutaminase. These observations suggest local synthesis of the neurotransmitter pool of glutamate at the synaptic vesicle, an efficient mechanism for vesicular glutamate refilling and, hence, glutamate transmission. Vesicle-bound AAT belongs to the mitochondrial type (GOT2) based upon kinetic and immunological properties [134] and proteomics analyses [109, 110]. Vesicle-bound AAT has much higher affinity for aspartate than does the cytosolic isozyme (GOT1). The aspartate K_m of the former is substantially lower than its cellular concentrations, whereas that of the latter is significantly higher. This suggests that vesicle-bound AAT would function at close to V_{max}. These findings indicate that α-ketoglutarate can effectively serve as an immediate precursor for synthesis of the neurotransmitter glutamate. Thus, vesicle-bound AAT could represent an efficient mechanism for generating the vesicular pool of glutamate.

α-Ketoglutarate would be derived from neuronal mitochondria, where it is produced from glutamine-derived glutamate [129] or from glucose via the TCA cycle, or could be supplied from astrocytes [135], where de novo synthesis of α-ketoglutarate occurs via CO_2 fixation of pyruvate, followed by the TCA cycle [136–145]. Synaptosomes have been shown to be capable of taking up α-ketoglutarate [146–148, Takeda and Ueda, unpublished observations] as well as glutamine [114; Takeda and Ueda, unpublished observations].

Since the catalytic site of mitochondrial glutaminase is likely to be localized to the inner surface of the mitochondrial inner membrane [124–128], the glutamine–glutamate conversion would occur in the matrix, where glutamate, rather than directly released to the cytoplasm, is largely transformed to α-ketoglutarate by mitochondrial AAT [129, 133] and glutamate dehydrogenase [149–152]. A large part of α-ketoglutarate produced from glutamine and a small part of α-ketoglutarate from glucose could be transferred to the cytoplasm, as mentioned above. These lines of evidence taken together suggest that α-ketoglutarate, not glutamine, serves as the major *immediate* precursor for the neurotransmitter pool of glutamate. However, in view of immunocytochemical evidence suggesting that cytosolic AAT is associated with glutamatergic nerve terminals [153, 154], the

relative contribution of vesicle-bound versus cytosolic AAT to synthesis of the neurotransmitter pool of glutamate remains to be established.

Although the glutamate–glutamine cycle is thought by many to play the central role in supplying the precursor of the neurotransmitter glutamate, the synthesis of the glutamate responsible for spontaneous synaptic transmission remains less clear [77]. Kam and Nicoll [155] showed that vesicular release of glutamate from neurons in culture devoid of astrocytes occurs even in the absence of glutamine in the culture medium. Masson et al. [156] observed that genetic deletion of phosphate-activated glutaminase partially reduced but did not abolish evoked release, whereas it had little effect on spontaneous release of glutamate. These lines of evidence indicate that the glutamate–glutamine cycle may not play the exclusive role in providing the immediate precursor for synthesis of the neurotransmitter pool of glutamate, in particular that released spontaneously and responsible for generating the miniature excitatory postsynaptic current. This suggests alternative pathways for such a role. α-Ketoglutarate derived from glucose or some amino acids or odd chain fatty acids [145] via the TCA cycle could serve as such a precursor. Glutamate could also be formed from some amino acids [145]. Moreover, it is plausible that even the synthesis of vesicular glutamate derived from glutamine could well be mediated in large part by formation of α-ketoglutarate, as described above. A proposed effective mechanism for synthesis and vesicular loading of the neurotransmitter glutamate is shown in Fig. 2.

Glutamate Release Regulation Via Modulation of VGLUT Activity and Expression

Since VGLUT plays a pivotal role in glutamate synaptic transmission, it is likely to be subject to regulation. This regulation would be important in controlling neuronal communication. A number of VGLUT inhibitors have been reported [52, 62, 70, 94, 157–170, 172]. However, only three inhibitors have been shown capable of down-regulating the amount of exocytotically released glutamate: the vesicular transmitter uptake inhibitory protein factor (IPF) [164], Rose Bengal [165], and ketone bodies, in particular acetoacetic acid [70]. IPF is a potent VGLUT inhibitor with a high molecular weight and an elongated shape [160]. When incorporated into synaptosomes, it led to reduction of exocytotically released glutamate [164]. However, the physiological function of IPF is not known. Rose Bengal is also a potent VGLUT inhibitor and, being hydrophobic in nature, permeates the synaptosome plasma membrane. Thus, it was shown able to suppress the amount of glutamate released by exocytosis [165]. However, both IPF and Rose Bngal exhibited potent inhibition of vesicular uptake of GABA and serotonin as well [160, 164, 167, 171]. Thus, these agents proved non-specific to VGLUT.

Recently, Rose Bengal has been reported to attenuate glutamate synaptic transmission [173, 174]. However, the effective concentrations were much higher than in glutamate release [165]. Juge et al. [70] have shown that ketone bodies inhibit VGLUT2 by blocking Cl^- activation, and that acetoacetate, the most potent, is capable of abolishing depolarization-induced glutamate release at millimollar concentrations. Acetoacetate decreased not only the amplitude and but also the frequency of occurrence of mEPSCs. These ketone bodies were suggested responsible for the benefits of the ketogenic diet on controlling seizures [70]. Other α-keto acids found in the maple syrup urine disorder, such as α-keto-β-methyl-n-methyl valeric acid and α-ketoisocaproic acid, have also been reported to inhibit vesicular glutamate uptake by blocking the Cl^--activating site [175]. However, these agents appear to also compete with the glutamate transport site. All the VGLUT-inhibitory keto acids reported are endogenous substances, but not potent. The most potent, highly VGLUT-specific inhibitors known today are Trypan Blue [161] and Brilliant Yellow [172], but these are not membrane-permeable. Thus, membrane-permeable, potent VGLUT-specific inhibitors, which could specifically regulate glutamate release, remain to be developed.

Characteristics of Three Glutamatergic Synapses

There are many synapses for which glutamate acts as the transmitter. Of these we have selected three which have other important values including their physiology, pharmacology, and recent molecular biology. Evidence for glutamate as the transmitter at squid, crayfish, and mammalian CNS synapses is widely acknowledged. Briefly included here are some examples of acceptable evidence. The localization and content of glutamate in the giant synapse of squid [176] constitutes some of the strongest evidence. This is in the face of problems involving experiments with glutamate application to neurons, determining the glutamate reversal potential, and detecting release of glutamate. In crayfish muscle, the distribution and pharmacological properties of synaptic glutamate receptors provide strong support for glutamatergic synaptic transmission [177]. It is well accepted that the vast majority of fast synaptic responses in the vertebrate brain use glutamate.

Presynaptic Ca^{2+}

An important property of synapses is the requirement of Ca^{2+} for evoked release as first demonstrated at the frog neuromuscular junction [178, 179]. In early observations

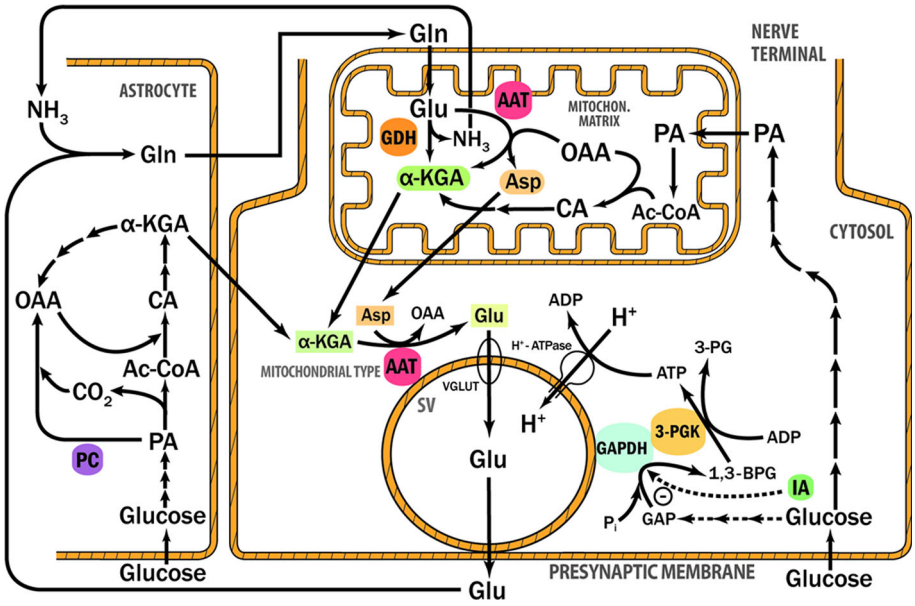

Fig. 2 Proposed effective mechanism for accumulating the neurotransmitter glutamate into synaptic vesicles. The VGLUT substrate glutamate is synthesized by vesicle-bound AAT from α-ketoglutarate and aspartate. Energy required for VGLUT's transport function is also generated by vesicle-bound, glycolytic ATP-generating enzymes, GAPDH and 3-PGK. The majority of α-ketoglutarate would originate from nerve terminal mitochondria, where it is produced from glutamine-derived glutamate by AAT and GDH; this glutamine–glutamate conversion would be carried largely out by glutaminase on the matrix side of the inner membrane. An additional source of α-ketoglutarate would be nerve terminal-linked astrocytes, where it is synthesized de novo via pyruvate carboxylation and the TCA cycle. Another minor source of α-ketoglutarate could be nerve terminal glucose, which leads to production of α-ketoglutarate via the TCA cycle. α-Ketoglutarate produced in this manner could serve as a substrate for synthesis of the transmitter glutamate responsible for spontaneous firing. This mechanism of supply of the VGLUT substrate does not depend on pre-neuronal activity in contrast to its supply derived from glutamine, whose synthesis in astrocytes heavily depends on glutamate released upon neuronal activation. Aspartate is supplied from mitochondria in the nerve terminal. *AAT* aspartate aminotransferase, *Ac-CoA* aceyl coenzyme A, *α-KGA* α-Ketoglutarate, *Asp* aspartate, *GAP* glyceraldehyde-3-phosphate, *GAPDH* glyceraldehyde-3-phosphate dehydrogenase, *GDH* glutamate dehydrogenase, *Glu* glutamate, *Gln* glutamine, *IA* iodoacetate, *OAA* oxaloacetic acid, *PA* pyruvic acid, *3-PG* 3-phosphoglycerate, *3-PGK* 3-phosphoglycerate kinase, *P* inorganic phosphate

on squid giant axons, Katz further demonstrated the importance of Ca^{2+} where strong depolarization of nerve terminals resulted in reduced transmission as the membrane potential reached the equilibrium potential for Ca^{2+} [180, 181]. Direct measurement of intracellular increase in Ca^{2+} was first shown in presynaptic terminals of the squid giant synapse with the Ca^{2+} detecting protein, aequorin [182]. Llinas et al. [183] used voltage clamp that characterized presynaptic Ca^{2+} currents and was able to demonstrate a minimal synaptic delay of 200 μs between a rapid influx of Ca^{2+} and the beginning of the postsynaptic response.

A classification scheme for Ca^{2+} channels (N-, T-, and L-types) was first described for sensory neurons [184] and then sympathetic neurons [185]. Interestingly, the N-type Ca^{2+} channels are coupled to norepinephrine release from the sympathetic neurons [186]. For most hippocampus glutamatergic neurons N-type [187] and P/Q-type [188, 189] Ca^{2+} channels contribute to synaptic transmission. The pharmacology of P-type Ca^{2+} channels indicates the release of glutamate at the squid giant synapse [190] and at the crayfish neuromuscular junction [191].

Ca^{2+} Dependence

Synaptic transmission requires Ca^{2+} for action potential evoked release [178] but not for spontaneous release [192, 193]. The Ca^{2+} appeared necessary to couple the action potential to the release process, but the final steps leading to exocytosis did not require Ca^{2+}.

The synaptic vesicle protein, synaptotagmin, in combination with RIM, Rim BP and Munc 13 have been hypothesized to be involved in Ca^{2+}-dependent release of neurotransmitter [194–196]. Synaptotagmin null mutants fail to have rapid synchronous neurosecretion but still respond to α-latrotoxin [195, 196].

Scheme of Synapsin Function

The role of Synapsin I in neurotransmitter (NT) release is well established and reviewed by Greengard et al. [200]. The hypothetical scheme of Synapsin I function is shown in Fig. 3. Synaptic vesicles (SV) are normally in a resting condition surrounded by bound Synapsin I. Synapsin I is

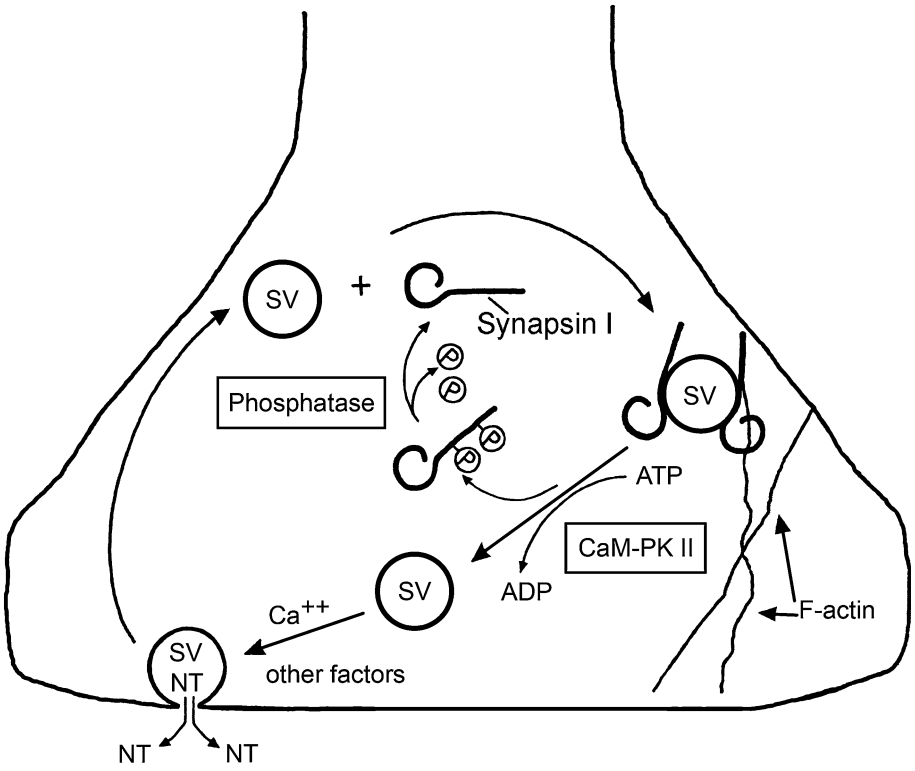

Fig. 3 Hypothetical scheme of Synapsin I function

also bound to F-actin, tethering synaptic vesicles to actin filaments and rendering the vesicles unavailable for release. Depolarization of the nerve terminal triggers an influx of Ca^{2+} and the activation of the calcium/calmodulin dependent protein kinase II (CaM-PK II). Phosphorylation (P) of Synapsin I by CaM-PK II decreases its affinity for the synaptic vesicles. The denuded vesicle can now be mobilized into a releasable pool. Activation of the vesicle by Ca^{2+} and other factors results in exocytosis and the release of neurotransmitter. The cycle is completed by the formation of new vesicles that are replenished with transmitter and recoated with dephospho-Synapsin I [201, 202].

SNARE Proteins

The SNARE hypothesis [194, 197–199] states that the presynaptic vesicle acts as a donor or agonist for a target molecular complex in the nerve terminal membrane (see Table 1). The docking and subsequent fusion events are thought to be mediated by the interaction of a protein complex consisting of cyctosolic proteins (SNAPs and NSF) with 3 synaptic proteins SNAP-25, Synaptobrevin and Syntaxin (the SNARE proteins). Munc 18 is a Syntaxin associated protein which acts to "clamp" the complex. Complexin binds to this partially assembled SNARE complex, generating the "superprimed" state. The v-SNARE Synaptobrevin is in the synaptic vesicle membrane while Syntaxin and SNAP-25 (t-SNARES) are in the presynaptic plasma membrane. The protein complex that recruits Ca^{2+} channels to active zones involves RIM, RIM-binding protein, and Munc 13 (a priming factor) interacting with Synaptotagmin. Ca^{2+} binding to synaptotagmin enables synaptotagmin to displace part of complexin bound to the SNARE complex, as well as to interact with plasma membrane phospholipid. This triggers vesicle-plasma membrane fusion pore opening, allowing initial neurotransmitter release, followed by complete membrane fusion. Thus, synaptotagmin mediates rapid coupling of action potential-induced Ca^{2+} influx to transmitter release.

In support of this hypothesis, injection of recombinant SNAPs into the giant presynaptic terminal of the squid enhances transmitter release [203]. Conversely, injection of peptides designed to mimic the sites at which SNAPs interacts with its binding partners inhibits transmitter release.

The Visualization of Vesicles in Living Preparations with Total Internal Reflectance Microscopy

First shown with exocytosis from chromaffin cells, Steyer et al. [204] used Total Internal Reflectance Fluorescence (TIRF) microscopy to demonstrate that dense core vesicles about 250 nm in diameter approach (24 nm/s, top speed

Table 1 Proteins related to presynaptic vesicular exocytosis

Process	Proteins	Activator	Proteolytic blockers
Mobilization	Synapsin	CaM-PK II	
Docking	Neurexin	Latrotoxin	
	SNAP 25		Botx A
	Synaptobrevin		Tetanus toxin
			Botx B,D,F, G
	Syntaxin		Botx C1
	Munc 18		
Docking, priming, Ca^{2+} channel recruitment	RIM	Rab 3a	
	RIM-BP		
	Munc 13		
Superpriming	Complexin		
Fusion	Synaptotagmin	Ca^{2+}	

Taken from Refs. [194, 197–200]

114 nm/s) and fuse with the plasma membrane (0.8 sites/μm^2). The technique relies on a laser beam that is directed at the interface between a glass coverslip and overlying solution at an angle that results in total reflectance of the beam back into the glass. An evanescent layer develops in the solution about 200 nm above the coverslip. The technique circumvents the resolution limits of bright field optics (200 nm). In both chromaffin cells and bipolar neurons [204, 205] repetitive stimulation results in reduced neurosecretion (fewer released vesicles).

Synaptic Plasticity: Persistent and Cumulative Neurotransmitter Release

Chemical synaptic transmission is not a static process. The release of neurotransmitter may increase or decrease depending upon the past history of the presynaptic activity. The examples given here are in well studied glutamatergic synapses: crustacean neuromuscular junction, squid giant synapse, and hippocampal synapses.

Short Term Synaptic Plasticity

1. Depression can be explained simply as a loss of the presynaptic vesicular store of neurotransmitter following repetitive stimulation. Also, depression can be reversed by rest.
2. Facilitation of neurosecretion elicited by repetitive stimuli is characterized by an early phase and a late phase. This is true of frog neuromuscular junction [206], squid giant synapses [207], and crayfish neuromuscular junctions [208–210].

   Frequency facilitation is a process whereby the quantal content increases as a function of repetitive stimulation. Analysis of the frequency dependence of the presynaptic parameters n (number of quanta capable of responding to a nerve impulse) and P (average probability of responding) has revealed the physiological basis underlying facilitation. Recent evidence suggests that the amount of glutamate per vesicle could alter P [211]. Since both n and P increase as a function of frequency, facilitation has been described in terms of the rates of quantal mobilization and demobilization [212, 213].

   Facilitation requires Ca^{2+} influx into the presynaptic terminal [214], and they proposed that facilitation at the neuromuscular junction is due to a residue of Ca^{2+} that enters during nerve stimulation. Strong support for the residual calcium theory came from experiments in which intracellular calcium was reduced by photolabile Ca^{2+} [215].
3. A more complete description of persistent effects was given by Magelby [216] in which enhanced release included facilitation as well as augmentation and potentiation (see Table 2). These forms of persistent neurosecretion were also dependent on residual Ca^{2+} [215]. Moreover, these different forms of release appear to require Ca^{2+} at different sites. The molecular mechanisms of how and where Ca^{2+} acts are still unresolved.

Synaptic Plasticity: Long-Term Persistent and Cumulative Release

1. Long term facilitation (LTF) at crustacean neuromuscular junctions is independent of Na^+ or Ca^{2+} currents

Table 2 Cumulative release of neurotransmitter

	Facilitation	Augmentation	Potentiation
Conditioning train	3 and up	10 and up	300 impulses
Decay time constant	F1 = 60 ms, F2 = 400 ms	~10 s	~1 min
Calcium ion entry	Yes	Yes	Yes
Pharmacology	Sr increases	Ba increases	

in presynaptic terminals. Experiments in which an inhibitor of adenylate cyclase was injected into nerve terminals blocked LTF [217]. Nerve impulses are somehow coupled to adenylate cyclase and the generation of cAMP which in turn activates protein kinase A and the enhancement of synaptic transmission.

2. The discovery of hippocampal long term potentiation (LTP) by Bliss and Lomo [218] is significant. Previously Ramon y Cajal [1] suggested that learning and memory could take place at synapses in the brain. Stimulation of a specific pathway at high frequency can cause an increase in synaptic strength that lasts over 10 h. In a search for sites and mechanisms of action, differential effects were obtained, dependent on the particular pathway activated [219]. Repetitive activation of the mossy fiber input to CA3 pyramidal cells will cause LTP without postsynaptic depolarization. Stimulation of other pathways (Schaffer collateral fibers, commissural fibers, and perforant path fibers) evoke LTP but only with concurrent depolarization of the postsynaptic neuron. The postsynaptic action involves released glutamate which activates both AMPA and NMDA receptors [219]. Current initially flows through the AMPA receptors but not through NMDA receptors because those receptor channels are blocked by Mg. Depolarization of the postsynaptic neuron removes the Mg block of the NMDA receptor and allows Na^+ and Ca^+ to enter the cell. Some aspects of LTP may be due to retrograde action back to the presynaptic glutamatergic terminals [219].
3. Long term depression occurs at cortical and cerebellar excitatory synapses. Similar to LTP, LTD involves postsynaptic elevation of intracellular calcium.

Astrocytic Release of Glutamate

Although the focus of this review is on the release of glutamate from nerve terminals, it is interesting that considerable effort has been placed on the release of glutamate from astrocytes. There are many properties of glutamate release from astrocytes that are similar to what is discussed above and it should be added that many of these are controversial [220]. It appears too early to discount these findings, but their function related to neurons is rather difficult to understand in terms of time [155] and space. Neuronal signaling relies on fast and precise timing as well as intricate and detailed anatomy. Perhaps astrocytic function will have a broad effect yet to be established.

Conclusion

Preparation for excitatory synaptic transmission in the vertebrate brain and spinal cord begins with transmitter synthesis and loading into synaptic vesicles. A large body of evidence now indicates that glutamate is specifically accumulated into synaptic vesicles, mediated by VGLUT at the expense of an electrochemical proton gradient generated by vesicular proton-pump ATPase. This provides strong support for the concept that glutamate is released from synaptic vesicles and plays a major role as an excitatory neurotransmitter.

Evidence suggests that vesicle-bound ATPase largely harnesses ATP generated via glycolysis, particularly by vesicle-bound GAPDH/3-phosphoglycerate kinase and pyruvate kinase. Other evidence suggests that synaptic vesicles are also capable of synthesizing glutamate by vesicle-bound AAT (of the mitochondrial type, distinct from the cytosolic isozyme) from α-ketoglutarate and aspartate. Both α-ketoglutarate and aspartate would be derived largely from mitochondria in the nerve terminal. α-Ketoglutarate synthesized de novo via pyruvate carboxylation could also be supplied from adjacent astrocytes. The majority of α-ketoglutarate originating in the mitochondria would be generated from glutamine-derived glutamate. Some may be a glucose-derived TCA cycle product.

These observations support the notion that synaptic vesicles, in order to sustain the neurotransmitter pool of glutamate, are endowed with an efficient mechanism for vesicular filling of glutamate. A proposed mechanism for synthesis of the neurotransmitter pool of glutamate is shown above in Fig. 2. However, in view of the occurrence of glycolytic ATP-generating enzymes and AAT in the cytosol, the relative contribution of the vesicle-bound versus cytosolic enzymes remains to be determined. Also to be developed are membrane-permeable, potent VGLUT-specific inhibitory agents, which could regulate glutamate release and synaptic transmission.

Glutamate-loaded vesicles undergo removal of Synapsin I by CaM kinase II-mediated phosphorylation, transforming to the release-ready pool. Vesicle docking to and fusion with the presynaptic plasma membrane are thought to be mediated by the SNARE complex. The Ca^{2+}-dependent step in exocytosis is proposed to be mediated by synaptotagmin.

Our aim was to review the processes of glutamate release from both biochemical and neurophysiological points of view. The accumulation of this knowledge has the benefit of providing a basis for understanding important mechanisms in space and time.

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