Permanent dynamic transporter-mediated turnover of glutamate across the plasma membrane of presynaptic nerve terminals: arguments in favor and against

Abstract: Mechanisms for maintenance of the extracellular level of glutamate in brain tissue and its regulation still remain almost unclear, and criticism of the current paradigm of glutamate transport and homeostasis has recently appeared. The main premise for this study is the existence of a definite and non-negligible concentration of ambient glutamate between the episodes of exocytotic release in our experiments with rat brain nerve terminals (synaptosomes), despite the existence of a very potent Na⁺-dependent glutamate uptake. Glutamate transporter reversal is considered as the main mechanisms of glutamate release under special conditions of energy deprivation, hypoxia, hypoglycemia, brain trauma, and stroke, underlying an increase in the ambient glutamate concentration and development of excitotoxicity. In the present study, a new vision on transporter-mediated release of glutamate as one of the main mechanisms involved in the maintenance of definite concentration of ambient glutamate under normal energetical status of nerve terminals is forwarded. It has been suggested that glutamate transporters act effectively in outward direction in a non-pathological manner, and this process is thermodynamically synchronized with uptake and provides effective outward glutamate current, thereby establishing and maintaining permanent and dynamic glutamateᵢᵓ/glutamateᵢᵓgradient and turnover across the plasma membrane. In this context, non-transporter tonic glutamate release by diffusion, spontaneous exocytosis, cystine-glutamate exchanger, and leakage through anion channels can be considered as a permanently added 'new' exogenous substrate using two-substrate kinetic model calculations. Permanent glutamate turnover is of value for tonic activation of post/presynaptic glutamate receptors, long-term potentiation, memory formation, etc. Counter-arguments against this mechanism are also considered.

Keywords: brain nerve terminals; glutamate extracellular level; glutamateᵢᵓ/glutamateᵢᵓgradient; glutamate turnover; non-pathological transporter-mediated glutamate release.

Rationale

Chemical synapses are the main structures responsible for nerve signal transmission in the brain. The basic principles of brain functioning still remain almost uncertain despite great efforts and a lot of experimental data. In this context, it is suggested that some misevaluation of the role of the key processes underlying synaptic transmission takes place. The main premise for this work is the fact that definite ambient concentrations of small amino acid neurotransmitters exist in presynaptic rat brain nerve terminals (synaptosomes) in our experiments albeit potent neurotransmitter's reuptake. Synaptosomes retain all characteristics of intact nerve terminals, that is, the ability to maintain membrane potential and accomplish uptake and transporter-mediated release of glutamate, exocytosis, etc., and are one of the best systems to explore the relationship between the structure of proteins, its biochemical and cell-biological properties, and physiological role (Südhof, 2004).

The extracellular level of glutamate in nerve terminals

Eighty years ago, it has been accepted that glutamate plays a central metabolic role in the brain (Krebs, 1935;
Chemical synapses release neurotransmitters by exocytosis of synaptic vesicles. Nerve signal transmission is initiated by depolarization of the plasma membrane of presynaptic nerve terminals followed by Ca\(^{2+}\) entrance, fusion of synaptic vesicles with the plasma membrane, and exocytotic release of the neurotransmitters to the synaptic cleft, which then interact with the postsynaptic receptors and activate the signaling pathways. After exocytosis, termination of neurotransmission occurs by neurotransmitter reuptake to the neurons and glial cells. It is so because there are no enzymes in the synaptic cleft responsible for degradation of glutamate [\(\gamma\)-aminobutyric acid (GABA), glycine, aspartate, etc.]. Glutamate transporters are integral membrane proteins with eight putative transmembrane domains, which locate in the plasma membrane of neurons and glial cells, and use Na\(^+/K\)-electrochemical gradient as energy source for transport of glutamate from the synaptic cleft into the cytosol against its gradient (Danbolt, 2001, for review; Kasatkina and Borisova, 2010). \(K_m\) values of glutamate uptake reported for brain synaptosome preparations vary between 1–5 μM, 13.9 μM, 25 μM, and 36 μM (Robinson, 1998; Debler and Lajtha, 1987; Bennett et al., 1973; Logan and Snyder, 1972; Danbolt, 2001, for review). In our experiments with synaptosomes, \(K_m\) was equal to 10.7±2.5 μM, and \(V_{\text{max}}\) was 12.5±3.2 nmol/min/mg of protein (Borisova et al., 2004; Borisova and Himmelrech, 2005). The glutamate binding sites in the transporters are optimized for binding of extracellular but not intracellular glutamate, and the affinity of the transporters for glutamate, when the substrate binding site faces the extracellular space, is about 50 times higher as compared to the intracellular glutamate binding, thereby promoting dissociation of glutamate into the cytoplasm against a high intracellular glutamate concentration (Watzke and Grewer, 2001). The roles of glutamate transporters in synaptic transmission are far from being completely understood.

The ambient concentration of glutamate between the episodes of its exocytotic release is a crucial parameter of synaptic neurotransmission. The mechanisms of its establishment and maintenance were assessed in a restricted number of publications. It is commonly accepted that the extracellular glutamate level is a balance between neurotransmitter uptake and release. There is a ‘dual’ contradicted significance of the extracellular level of glutamate in the synaptic cleft. From one side, a low extracellular glutamate concentration maintained between episodes of exocytotic release prevents continual activation of postsynaptic glutamate receptors and protects neurons from excitotoxic injury and death and also provides a high signal-to-noise ratio for glutamatergic transmission (Jabaudon et al., 1999; Cavelier et al., 2005). From the other side, the certain level of ambient glutamate is very important for tonic activation of presynaptic and postsynaptic glutamate receptors, e.g. NMDA receptors in hippocampal slices, AMPA, kainite, and mGlu receptors, which is a way of regulation of glutamatergic transmission (Sah et al., 1989; Dalby and Mody, 2003; Cavelier and Attwell, 2005), and knowledge of the precise ambient extracellular glutamate concentration in the brain is required for understanding its potential impacts on tonic and phasic receptor signaling (Suna et al., 2014). Definite extracellular neurotransmitter concentration in vivo is significant for several neurological processes such as migration and differentiation of brain cells during development and synaptic plasticity (LoTurco et al., 1995; Nguyen et al., 2001; Manent and Represa, 2007; Featherstone and Shippy, 2008).

A lack of consensus regarding the value of the extracellular glutamate level is observed among groups (Pendyam et al., 2012). The existence of numerous nonlinear mechanisms makes the characterization of glutamate homeostasis in the synapses very difficult (Pendyam et al., 2012), and the concentration of glutamate in the central nervous system varies in dependence on the biological compartment being measured (Moussawi et al., 2011). Glutamate levels in cerebrospinal fluid are around 10 μM; extracellular glutamate concentrations in the brain are approximately 10 nM (Danbolt, 2001, for review; Featherstone and Shippy, 2008). Glutamate concentration in the synaptic cleft following action potential-mediated release exceeds 1 mM for <10 ms and quickly returns to <20 nM between release events due to glutamate uptake by neurons and glia (Dzubay and Jahr, 1999). The extracellular glutamate concentration that resulted from electrophysiological estimates of tissue slices in vitro was 0.02–0.1 μM, whereas in vivo measurements using microdialysis or voltammetry varied between 1 and 30 μM (Moussawi et al., 2011).
Thus, estimates of the basal concentration of glutamate within the extracellular space vary over three orders of magnitude, ranging from 0.02 to 30 μm, and depend on the methods of measurement (Herman and Jahr, 2007; Chefer et al., 2009; Moussawi et al., 2011). Estimates of ambient glutamate based on microdialysis measurements are approximately 100-fold higher than those based on electrophysiological measurements of tonic NMDA receptor activity (Suna et al., 2016). It is so because a thin layer of damaged tissue with disrupted glutamate transport could underlie the significant quantitative discrepancy between the ambient glutamate estimates provided by electrophysiological studies in slices and those from microdialysis studies (Suna et al., 2014). For monoamine-, acetylcholine-, and purine-type neurotransmitters, the extracellular concentrations are also maintained at low levels (<100 nm); however, it is determined presumably by neurotransmitter release (van der Zeyden et al., 2008).

The mechanism maintaining correct extracellular level and homeostasis of glutamate in the central nervous system and its regulation is crucial for understanding normal and pathological synaptic neurotransmission, and the absence of consensus in estimations of ambient glutamate concentration reveals the multiplicity of these mechanisms. Several publications with critics of the current paradigm of glutamate transport and homeostasis have recently appeared (Funicello et al., 2004; Pendyam et al., 2012; Makarov et al., 2013). In our experiments, an increase in the ambient level of glutamate under conditions of cholesterol deficiency was associated with weak uptake in nerve terminals, and also tonic (Borisova et al., 2010a, b) and transporter-mediated (Krisanova et al., 2012) release of L-[14C]glutamate depended on the ratio of glutamate_in/glutamate_out in nerve terminals. However, changes in transporter-mediated uptake of glutamate were not always accompanied by alterations in the ambient level of glutamate that was shown under specific conditions of centrifuge-induced hypoxia (Borisova et al., 2004; Borisova and Himmelreich, 2005; Borisova and Krisanova, 2008; Krisanova et al., 2009). Average basal extracellular concentration of L-[14C]glutamate in L-[14C]glutamate-preloaded synaptosomes in our experiments was equal to 0.193±0.013 nmol/mg protein (Borisova et al., 2010a). This ambient level of L-[14C]glutamate estimated as percentage of total label incorporated in nerve terminals consisted of 18%±1% and was similar in Ca²⁺-free and Ca²⁺-containing media and depended on synaptosomal protein concentration and so on the number of glutamate transporters in the preparation (Borisova et al., 2010a). The dependence of ambient glutamate on membrane densities of expressed glutamate transporters was shown in Xenopus oocytes (Suna et al., 2014).

According to the existing theory, the establishment and maintenance of the extracellular level of glutamate are determined by a balance between non-transporter tonic release (occurred through diffusion, spontaneous exocytosis, anion channels, cystine-glutamate exchanger (Bouvier et al., 1992; Danbolt, 2001, for review; Cavelier and Attwell 2005) and uptake of glutamate that can be represented as follows:

\[
\text{[glu]_{out}} = [\text{glu}]_{\text{non-transporter tonic release}} - [\text{glu}]_{\text{uptake blocked}}
\]

To maintain ambient L-[14C]glutamate at the permanent level of 18%±1% of total label incorporated in nerve terminals, tonic non-transporter release should significantly exceed the uptake of L-[14C]glutamate. However, in our experiments, tonic non-transporter release of L-[14C]glutamate in the presence of glutamate transporter inhibitor DL-threo-β-benzylxoxyspartate (DL-TBOA) (200 μm) consisted of 7.8%±0.6% of total label for 6 min and was accompanied with a consequent increase in extracellular L-[14C]glutamate (Borisova and Krisanova, 2008; Borisova et al., 2010a). So, tonic release during blockage of glutamate transporters is at least two times lower than the extracellular level of L-[14C]glutamate in synaptosomes, which can be represented as follows:

\[
\text{[glu]_{out}} > [\text{glu}]_{\text{non-transporter tonic release}} + [\text{glu}]_{\text{uptake blocked}}
\]

Therefore, there is no balance between the extracellular level of L-[14C]glutamate and its tonic release from synaptosomes under conditions of blocked glutamate transporter functioning. So, the contribution of transporter-mediated release to the establishment of the extracellular level of glutamate can be predicted (see the next sections).

The absolute value of the ambient level of glutamate in the parallel synaptosomal preparations was determined in our experiments by different methods, that is, amino acid analyzer, spectrofluorimetric glutamate dehydrogenase assay, and amperometric glutamate-sensitive biosensor based on the analysis of the activity of glutamate oxidase, and it was equal to 2.8 μm (Borisova et al., 2010a; Borisova, 2013), 2.3 μm (Krisanova et al., 2012), and 79 μm (Soldatkin et al., 2015), respectively. A comparative study of glutamate uptake using L-[14C]glutamate and glutamate biosensor (Soldatkin et al., 2015) revealed that almost all exogenously added glutamate (10 μm) was utilized by glutamate transporters for several minutes. Similarly with abovementioned experiments with radiolabeled L-[14C]glutamate, it is difficult to explain the fact that the extracellular level of glutamate in our biosensor experiments was almost similar (≈8 μm) before the addition of exogenous glutamate (10 μm) for initiation.
of uptake process and after completion of uptake. So, a constant amount of ambient glutamate remains in the medium before and after uptake measurements (stimulated by the addition of exogenous neurotransmitter at a concentration of 10 μM).

Permanent glutamate turnover across the plasma membrane of nerve terminals

As it can be concluded from the abovementioned section, it is absolutely unclear why definite and non-negligible concentration of extracellular glutamate exists in our experiments and why ambient glutamate is not deleted from the extracellular space, despite the availability of strong and potent mechanism of transporter-mediated uptake. Also, there is no balance between non-transporter tonic release and the extracellular level of L-[14C]glutamate in our experiments (Equation 2). In this context, use of isolated nerve terminals allows to overcome contribution of glial uptake, thereby uncovering and bringing new insight to the mechanisms of the establishment of ambient glutamate level.

The abovementioned facts and the existence of definite level of ambient L-[14C]glutamate (Borisova et al., 2010a) and also of definite extracellular level of endogenous glutamate in nerve terminals determined by amino acid analyzer, spectrofluorimetric glutamate dehydrogenase assay, and amperometric glutamate-sensitive biosensor in our experiments (Borisova et al., 2010a; Krisanova et al., 2012; Soldatkin et al., 2015) forced the author to forward new hypothesis. The existence of the efficient permanent extracellular/intracellular glutamate exchange and so the permanent dynamic glutamate gradient across the plasma membrane of nerve terminals and the central role of glutamate transporters in this glutamate turnover is suggested. As glutamate transporter functioning is driven by Na+/K+ electrochemical gradient across the plasma membrane created by Na/K ATPase, it is clear that the maintenance of such permanent glutamate gradient requires a significant energy supplementation.

The maintenance of a permanent glutamate gradient and turnover across the plasma membrane requires specific mechanisms providing both the inward and outward glutamate current in nerve terminals. Below, we discuss potency, strength, and weakness of all prospective candidates ensuring the direct and reverse glutamate current in presynapse.

Suggested mechanisms for permanent inward glutamate transport in nerve terminals

This is a formal subsection concerning inward glutamate transport mechanisms, because between three possible candidates, that is, high-affinity Na+-dependent glutamate transporters, low-affinity glutamate transporters, and glutamate-glutamine neuronal-glial cycle, only high-affinity Na+-dependent glutamate transporters in nerve terminals are able to provide effective, highly regulated permanent inward movement of glutamate molecules across the plasma membrane. It is commonly accepted that, namely, Na+-dependent glutamate transporters provide the maintenance of low extracellular glutamate concentration in the synaptic cleft. Low-affinity uptake exhibits $K_m$ values above 500 μM (Johnston, 1981). This uptake system has been suggested to supply brain cells with amino acids for metabolic purposes (for review, see studies by Erecinska and Silver [1990] and Danbolt [2001]). In vivo, high-affinity Na+-dependent glutamate transporters of glial cells and glutamate-glutamine cycle can also contribute to inward glutamate transport. The role of glia in the maintenance of proper extracellular glutamate level is to delete an excess of glutamate from the extracellular space and to prevent uncontrolled leakage of glutamate from the synaptic cleft to the intersynaptic space (see Danbolt, 2001, for review).

Suggested mechanisms for permanent outward glutamate transport in nerve terminals

The most complicated issue is to specify the potential mechanisms providing permanent outward glutamate current in nerve terminals. One of the main requirements is that this process should be potent enough as compared to uptake system, be highly regulated, and reacts immediately on changes in the functional state of nerve terminals. It is clear that any misregulation in outward glutamate current can lead to serious consequences impairing neuronal communication. The suggested mechanisms maintaining permanent outward glutamate transport from nerve terminals are the following: (1) non-pathological transporter-mediated release of glutamate synchronized with uptake, (2) spontaneous exocytosis, (3) diffusion, (4) cystine-glutamate exchange, (5) release through anion channels, and also glutamate release as consequence of
activation of presynaptic glutamate receptors. Below, we examined the strong and weak points of all these candidates for outward glutamate current.

**Non-pathological transporter-mediated glutamate release synchronized with uptake**

It is accepted that glutamate transporter reversal is the main mechanism of glutamate release resulting in an increase in ambient glutamate and development of excitotoxicity under special conditions of energy deprivation, hypoxia, hypoglycemia, ischemia, brain trauma, stroke, etc. (Grewer et al., 2008).

Our data showed that ‘cold’ glutamate-, dl-threo-ß-hydroxyaspartate (dl-THA)- (Krisanova et al., 2012), and ß-aspartate (unpublished data) substituted L-[14C]glutamate in synaptosomes and induced a comparable increase in the ambient level of L-[14C]glutamate (and also D-[1H]aspartate) in nerve terminals. So, a quick redistribution of L-[14C]glutamate between intracellular and extracellular spaces, the new intracellular/extracellular ratio of glutamate/L-[14C]glutamate, and efficient synaptosomal extracellular/intercellular glutamate turnover take place in nerve terminals. The common feature of all studied substances, i.e. glutamate, dl-THA, and ß-aspartate, is that they are substrates of glutamate transporters. The resemblance of the data obtained with above agents, some of which are the receptor agonists or the substrates for different enzymes and exchangers, shows that registered dynamic changes in extracellular/intracellular distribution of radioactive L-[14C]glutamate are due to the functioning of glutamate transporters only. It is hypothesized that glutamate transporters provide dynamics extracellular/intracellular L-[14C]glutamate turnover after the application of different transporter substrates. The similarity in the effectiveness of different transporter substrates in the establishment of new extracellular level of the neurotransmitter indicates the central role of the transporters in this efficient glutamate exchange between the extracellular and intercellular compartments of nerve terminals.

Cavaliere and Attwell (2005) considered a possibility that exogenously applied glutamate might act on cell in the slice to release further glutamate. Recently, Makarov et al. (2013) have proposed a complex two-substrate kinetic model that takes into account transporter reversal. It has been suggested that the Michaelis-Menten kinetic scheme, which is popular in transporter studies, does not include transporter reversal and completely neglects the possibility of equilibrium between the substrate concentrations on both sides of the membrane. According to Makarov et al. (2013), one transporter substrate can release another one already accumulated inside the cell. This model allows the calculation of a ‘heteroexchange’ and ‘transacceleration’ using standard Michaelis coefficients for respective substrates. Transport depends on the energy balance of coupled transport of the substrate and simultaneously transported ions. The main conclusion of Makarov et al. (2013) is that when the substrates in both sides of the membrane are in equilibrium, the addition of extra substrate leads to re-equilibrium and release of the substrate (the same one already accumulated in the cytosol) from the cell.

In the present study, a new vision on glutamate transporter functioning in non-pathological reverse mode is forwarded. The author suggests that glutamate transporters act effectively in reverse direction in non-pathological manner; this process is thermodynamically synchronized with uptake, providing effective outward glutamate flow from nerve terminals, thereby maintaining permanent and dynamic glutamate\textsubscript{in}/glutamate\textsubscript{out} gradient across the plasma membrane and supporting the establishment and maintenance of a definite extracellular level of the neurotransmitter. Equation (1) can be corrected as follows: \[
\text{[glu]\textsubscript{out}} = \text{[glu}\text{non-transporter tonic release}] + \text{[glu}\text{uptake}} + \text{[glu}\text{non-pathological transporter-mediated release}}
\]

**Spontaneous exocytosis of synaptic vesicles**

Despite the selection of abovementioned candidate responsible for effective outward glutamate flow, that is, non-pathological transporter-mediated release of glutamate, there are other candidates for this position, which cannot be excluded from consideration. These mechanisms should afford efficient and regulated release of glutamate, and several more or less eligible candidates, e.g. diffusion, spontaneous exocytosis, cystine-glutamate exchanger, and release through anion channels, are analyzed below.

Neuronal communication is provided by precisely timed stimulated compound exocytosis of synaptic vesicles containing neurotransmitters, which is triggered by an intracellular Ca\textsuperscript{2+} spike that follows action potentials (Südhof, 2004; Rizzoli, 2014). Synaptic vesicles are subsequently retrieved from the plasma membrane of the presynaptic terminals, that is, endocytosis, and are turned into new fusion-competent synaptic vesicles (synaptic vesicle recycling). Rizzoli (2014) has underlined that synaptic vesicle recycling is one of the best controlled processes in cell biology; however, it is still unclear how
this process is controlled. A special case of fusion of synaptic vesicles is the ‘kiss and run’ mechanism, in which the vesicles fuse only transiently with the plasma membrane, release their content to the extracellular space via fusion pore, after which the fusion pore is closed (Alabi and Tsien, 2013). The evidence in favor of the existence of ‘kiss and run’ mechanism presented over the last decade has been subsequently argued against on technical grounds (Rizzoli, 2014).

In parallel to stimulated exocytic release, all synapses also exhibit spontaneous release of neurotransmitters (Truckenbrodt and Rizzoli, 2014; Kavalali, 2015). The major difference between these two mechanisms of the release is that stimulated exocytosis is directly coupled with action potentials, while spontaneous release is suggested to take place without any triggers. Spontaneous release of neurotransmitters occurs in the absence of stimulation in virtually all synaptic boutons (Truckenbrodt and Rizzoli, 2014). Spontaneous release can be increased by Ca\(^{2+}\)-dependent manner, e.g. caffeine-triggered Ca\(^{2+}\) release from internal stores (Zefirov et al., 2006), and by Ca\(^{2+}\)-independent way, e.g. by the application of lanthanum ions (Heuser and Miledi, 1971), α-latrotoxin (Ceccarelli et al., 1973; Betz and Henkel, 1994), or hyperosmotic sucrose (Rosenmund and Stevens, 1996; Truckenbrodt and Rizzoli, 2014). It has long been thought that this exocytosis is driven by fluctuations in local Ca\(^{2+}\) levels. The vesicles responding to these fluctuations are suggested to be the same ones that release upon stimulation, albeit potentially triggered by different Ca\(^{2+}\) sensors. This view has been challenged by several recent works, in which it has been suggested that spontaneous release is driven by a separate pool of synaptic vesicles (Truckenbrodt and Rizzoli, 2014). Many proteins, which constitute the core of fusion machinery, are required for exocytotic process, that is, SNAREs syntaxin 1, SNAP-25, and synaptobrevin. Holt et al. (2008) found that synaptic vesicles are constitutively active fusion machines, which function independently of Ca\(^{2+}\)-needing only synaptobrevin for activity. The final step in fusion does not involve the regulatory activities of other vesicle constituents, although these may be involved in regulating earlier processes (Holt et al., 2008). Taking into account the abovementioned facts, spontaneous exocytosis cannot be excluded from consideration as one of the candidates ensuring permanent outward glutamate flow. However, Jabaudon et al. (1999) have demonstrated that tonic glutamate release does not reflect potential-evoked and spontaneous exocytosis in cultural hippocampal slices because it is not blocked by TTX, botulinum, and tetanus neurotoxins.

Compartmentalization of glutamate in synaptic vesicles gives an excellent opportunity to ‘mask’ the major part of cytosolic neurotransmitter, thereby withdrawing vesicular glutamate at least partially from thermodynamic reliance of glutamate transporters. Glutamate transporters work only with existent extracellular and cytosolic pool of glutamate performing effective glutamate turnover, followed by the establishment of definite glutamate gradient. However, it should be underlined that the process of glutamate uptake by synaptic vesicles should be also taken into consideration in calculations.

Other mechanisms of non-transporter tonic release

A cystine-glutamate antiporter/exchanger carries cystine into the cell in exchange of internal glutamate (Bannai, 1986) and couples the uptake of one molecule of cystine to the release of one molecule of glutamate (Bannai, 1986; Baker et al., 2003; Bridges et al., 2012a,b). The physiological role of this exchanger is to act as a cystine transporter that uses the transmembrane gradient of glutamate as driving force. Extracellular glutamate inhibits cystine uptake, and when the extracellular concentration of glutamate became higher, the transporter may actually catalyze a release of cystine (see study of Danbolt [2001] for review). However, according to Cavelier and Attwell (2005), cystine-glutamate exchange does not generate tonic glutamate release. Theoretical calculations suggest that a significant fraction of tonic glutamate release in hippocampal slices could occur via diffusion of glutamate across the lipid membranes (Cavelier and Attwell, 2005); however, it is clear that this process is uncontrolled. 4,4′-Diisothiocyano-2,2′-stillbene-disulfonic acid, DIDS, has been found to block numerous anion transporters and channels, which also represent a potential way of outward glutamate current. According to Cavelier and Attwell (2005), tonic glutamate release is reduced by DIDS, and so it is mediated by DIDS-sensitive mechanism. In our experiments, the application of 1 mM DIDS for inhibition of anion exchange in nerve terminals did not significantly affect (approximately by 10%) the extracellular level of glutamate in synaptosomes (unpublished data).

Activation of presynaptic glutamate receptors by ambient glutamate can stimulate glutamate outflow from nerve terminals, resulting from receptor-dependent Na\(^{+}\) and Ca\(^{2+}\) entrance to the cytosol, activation of signaling pathways, ROS formation, etc. However, the application of NMDA (250 μM), AMPA (250 μM), and kainite (250 μM) did not increase significantly, e.g. by <15%, the extracellular
level of L-[14C]glutamate in nerve terminals in our experiments as compared to 100 μM glutamate-induced enlargement in this level, i.e. by 100% (unpublished data). The latest is a result of not only activation of presynaptic glutamate receptors but also transporter-mediated redistribution of the neurotransmitter between the intracellular and extracellular spaces.

Role of transporter-mediated glutamate release and non-transporter tonic release in outward glutamate current

The author hypothesizes that the abovementioned mechanisms of non-transporter tonic release of glutamate can be considered in two-substrate kinetic model of Makarov et al. (2013), as the addition of new exogenous substrate that changes the existing equilibrium of glutamate, which is maintained by glutamate transporters in both sides of the plasma membrane, and induces uptake of glutamate from the extracellular space and the consequent release of cytosolic glutamate by transporters. Kinetics of this possibility can be taken from following theoretical calculations, i.e. when the substrates in both sides of the membrane are in equilibrium, and then the addition of new portion of the substrate leads to re-equilibrium and release of the intrinsic ‘second’ substrate (Makarov et al., 2013). The abovementioned non-transporter mechanisms (together with spontaneous exocytosis) providing for glutamate leakage from nerve terminals and an increase in ambient glutamate can be also considered in kinetic calculations as ‘the new exogenous substrate’ and so cause simultaneous transporter-mediated uptake and release and thus re-equilibrium of glutamate between the extracellular space and the cytosol. It should be noted that in our experiments, the extracellular level of L-[14C]glutamate in nerve terminals under conditions of inhibition of glutamate transporters by 200 μM DL-TBOA increased by 10.8%±0.6% of total label accumulated by synaptosomes for 12 min (p≤0.05, Student’s t-test, n=4) (Borisova et al., 2010a). This value corresponds to net tonic glutamate release not only by spontaneous exocytosis of synaptic vesicles filled with glutamate but also by all possible mechanisms (cystine-glutamate exchanger, diffusion, anion channels), and so the amount of ‘the new exogenous substrate’ is non-negligible in kinetic calculations. Regulated exocytosis leads to the release of bulk glutamate to the synaptic cleft that also can be considered as the ‘the new exogenous substrate’ and so can cause a re-equilibrium of glutamate between the extracellular space and the cytosol resulting from simultaneous uptake and release of cytosolic glutamate by transporters. Also, it should be kept in mind that the local concentrations of glutamate at the transporter site could differ from those in the whole compartments.

Continuous non-transporter tonic release of glutamate in outer space of the plasma membrane and also glutamate uptake by synaptic vesicles inside of nerve terminals permanently disbalance transporter-dependent glutamate gradient across the plasma membrane, making this system very dynamic and dependent not only on Na+/K+ electrochemical gradient, and thus on the potential of the plasma membrane, but also on the functional state of synaptic vesicles and their ability for consequent uptake of cytosolic glutamate, efficiency of spontaneous exocytosis, lipid composition of the membranes, and thus plasma membrane permeability for glutamate, and effectiveness of diffusion, etc.

A kinetic algorithm at steady-state velocities can be presented as follows:

\[
Tr + [\text{glu}]_{\text{out}} \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} Tr \cdot \text{glu} \overset{k_2}{\underset{k_{2-1}}{\rightleftharpoons}} [\text{glu}]_{\text{in}}
\]  

(4)

where Tr is the glutamate transporters and glu is the L-glutamate.

A kinetic algorithm with consideration of permanent tonic glutamate release as new added substrate can be written as follows:

\[
[\text{glu}]_{\text{out \ originated}} \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} Tr \cdot \text{glu} \overset{k_2}{\underset{k_{2-1}}{\rightleftharpoons}} [\text{glu}]_{\text{in \ originated}} + Tr
\]

(5)

where Tr is the glutamate transporters and glu is the L-glutamate.

Accordingly, a kinetic algorithm with consideration of vesicular glutamate uptake can be written as follows:

\[
[\text{glu}]_{\text{vesicular}} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{Vesicular transporter} \cdot \text{glu} \overset{k_1}{\underset{k_{1-2}}{\rightleftharpoons}} \text{Vesicular transporter} + [\text{glu}]_{\text{vesicular}}
\]

(6)

Physiological significance of permanent glutamate turnover

The physiological importance of permanent intensive glutamate turnover across the plasma membrane and maintenance of definite glutamate concentration in the...
synaptic cleft (but at low levels) is underscored by several factors. First, the significance of proper extracellular level of glutamate for tonic activation of postsynaptic receptors has already been underlined in several publications (Sah et al., 1989; Dalby and Mody, 2003; Cavelier and Attwell, 2005), which may be of value for long-term potentiation and memory formation, making synaptic contacts permanently active. The regulatory role of activation of presynaptic glutamate auto- and hetero-receptors in the transport of glutamate and other neurotransmitters can be also considered. Second, permanent glutamate turnover makes nerve terminals very flexible and able to react immediately on the changes in their functional state, excess of exogenous glutamate, hypoxia, hypoglycemia, etc. Third, ambient glutamate is strongly dependent on the number of glutamate transporters expressed in the plasma membrane of nerve terminals (Borisova et al., 2010a; Suna et al., 2014), and so the concentration of glutamate in different synaptic clefts and extracellular spaces of the brain regions can be different and can correlate with glutamate transporter density and so may be one of the main regulatory mechanisms of brain functioning. The molecular mechanisms regulating transporter intracellular trafficking have been identified (González et al., 2002), and surface diffusion of astrocytic glutamate transporters and the fact that GLT-1 transporters are highly mobile on rat astrocytes have been recently demonstrated (Murphy-Royal et al., 2015).

The suggestion on the existence of a permanent dynamic gradient of neurotransmitters can be actual for other secondary-active neurotransmitter transporters, e.g. GABA, glycine, and possibly dopamine, whereas the physiological significance of these gradients is different from that of glutamate. It is so because in our experiments, the extracellular level of \([^{3}H]GABA\) in synaptosomes is also maintained at a definite and non-negligible level (Pozdnyakova et al., 2014; Borisova et al., 2015). The extracellular level of \([^{14}C]glycine\) is unstable, presumably because of its very intensive diffusion through the plasma membrane (unpublished data). The main uncertainty of the abovementioned suggestion is the fact that the author based it on her own experiments performed in isolated rat brain nerve terminals. However, the use of nerve terminals allowed the author to avoid consideration of glial processes and to concentrate attention on presynaptic nerve terminals only. Also, the author compared her own results obtained on synaptosomes with the data of other methodological approaches.

In conclusion, a new vision on non-pathological functioning of glutamate transporters in reverse direction

![Figure 1](image-url)
between the episodes of exocytotic release was forwarded, in addition to the previous one, where glutamate transporter reversal was considered as the main mechanism of glutamate release resulting in an increase in ambient glutamate and development of excitotoxicity under special conditions of energy deprivation, hypoxia, hypoglycemia, brain trauma, stroke, etc. It has been suggested that glutamate transporters act effectively in outward direction, and this process is thermodynamically synchronized with uptake and provides glutamate outflow, thereby establishing and maintaining permanent and dynamic glutamate\textsubscript{in}/glutamate\textsubscript{ex} gradient and turnover across the plasma membrane. In this context, glutamate enriching the extracellular space through tonic release by means of diffusion, spontaneous exocytosis, cystine-glutamate exchanger, and leakage through anion channels can be considered as permanently added exogenous substrate in two-substrate kinetic model calculation (Figure 1). Permanent glutamate turnover may be of value for tonic activation of postsynaptic/presynaptic glutamate receptors, long-term potentiation, memory formation, etc. This mechanism can be actual for secondary neurotransmitter transporters, e.g. GABA.

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