

THE INS AND OUTS OF GLUTAMATE TRANSPORTER PHARMACOLOGY



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Introduction

The presence of an extensive array of excitatory amino acid (EAA) ionotropic and metabotropic receptors is critical to the ability of the neurotransmitter L-glutamate to contribute to a spectrum of functions in the CNS that ranges from standard fast synaptic signaling to neuroplasticity to neurodegeneration.^{11,16,34,36,63} Another important element in this equation, however, is the amount of L-glutamate present in the synaptic environment and it is within this context that there is increasing interest in the glutamate transport systems. The capacity of these uptake systems to effectively sequester L-glutamate in glia, neurons and in synaptic vesicles is likely to be an important factor in the release, signal termination, and recycling of this excitatory neurotransmitter, as well as in the protection of neurons from excitotoxic injury.^{13,25,26,42,44,52,55} The goal of this brief review is to provide an update on a number of the pharmacological tools that are being

used to define and manipulate the function of these glutamate transporters.

Cellular excitatory amino acid transporters

While L-glutamate can act as a substrate of numerous cellular uptake systems, the transporters accepted as playing the most significant role in relation to excitatory neurotransmission in the CNS are collectively referred to as the high-affinity, sodium-dependent systems. Harnessing transmembrane ion gradients as the driving force, these uptake systems are capable of maintaining intracellular concentrations of L-glutamate several thousand-fold above extracellular levels.⁶² Stoichiometric studies suggest that the intracellular translocation of one molecule of L-glutamate is coupled to the inward movement of 3 Na⁺ ions and 1 H⁺ ion and the outward movement of 1 K⁺ ion.^{31,62} Comparisons of substrate specificity (e.g. glutamate vs. homocysteate), differential inhibition (e.g. sensitivity to dihydrokainate), kinetics, cell type (e.g. neuron vs. glia) or anatomical location (e.g. cortical vs. cerebellar) in a variety of preparations all pointed to heterogeneity within the sodium-dependent transport proteins. A major step, not only resolving this issue, but in advancing our understanding of the structure, mechanism and physiological properties of the transporters, came with the cloning and expression of distinct transporter subtypes.^{29,43,50} Referred to as EAATs (used for the human clones as an acronym for Excitatory Amino Acid Transporters), at least 5 distinct subtypes have been identified that (along with the neutral amino acid transporters ASCT1 and ASCT2) appear to be part of a novel gene family.^{2-4,22} A conserved heptapeptide sequence [AA(I/Q)FIAQ] appears to be a defining structural motif of the family, with homology among the various

Table 1. Differentiation of the excitatory amino acid transporter

Subtype	Primary Distribution in Brain	Distinguishing Pharmacological Features
EAAT1 GLAST, <i>rat</i>	Cerebellar glia	4-MG and L-SOS as substrates
EAAT2 GLT1, <i>rat</i>	Forebrain glia	DHK, MPDC , L- <i>trans</i> -2,3-PDC and 3-TMG as non-transportable inhibitors
EAAT3 EAAC1, <i>rabbit</i>	Cortical neurons	L-aspartate-β-hydroxamate as an inhibitor
EAAT4	Cerebellar Purkinje neurons	L-α-AA as a substrate
EAAT5	Retina	THA and L- <i>trans</i> -2,4-PDC as non-transportable inhibitors

(Bold text denotes compounds available from Tocris)

Abbreviations include: 4-MG = (2S,4R)-4-methylglutamate; L-SOS = L-serine-O-sulfate; DHK = Dihydrokainic acid; 3-TMG = (±)-threo-3-Methylglutamic acid; L-α-AA = L-α-amino adipate; THA = β-threo-hydroxy-aspartate

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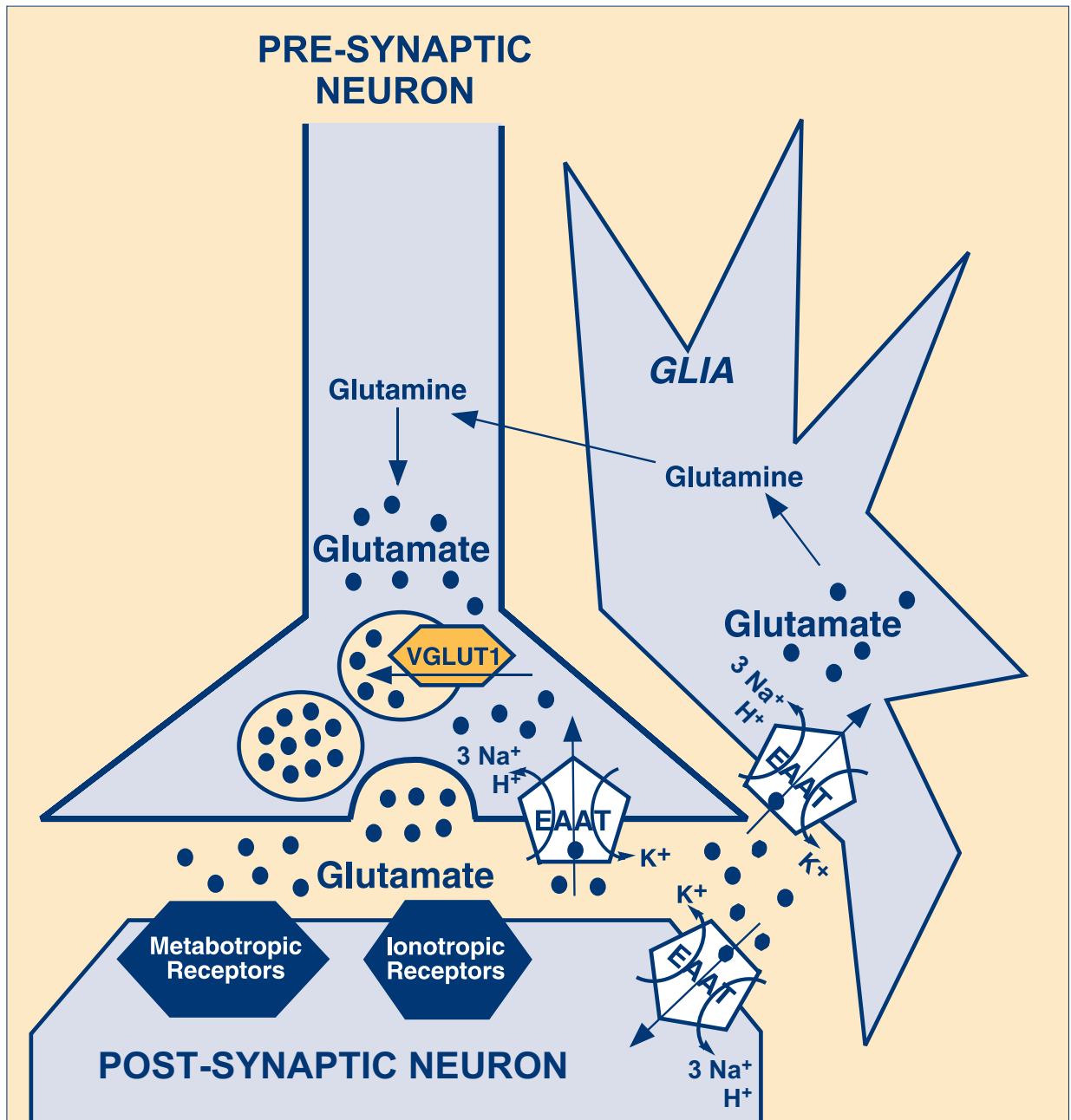
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transporters running at about 40-50%. As summarized in Table 1, EAAT1-5 exhibit distinct cellular and anatomical distribution, as well as marked differences in pharmacological specificity.

The presence of distinct EAAT subtypes naturally leads to questions regarding the possibility of subtype-specific roles and, consequently, to the need for selective inhibitors with which to probe function. Toward this goal, the cloning and expression of individual transporters in model systems (especially *Xenopus* oocytes) has not only provided a strategy for selective characterization, but has also allowed transport to be readily quantified by electrophysiological methods. As EAAT-mediated transport is electrogenic, uptake can be followed by measuring substrate-induced currents (see multiple chapters in ¹). Indeed, substrate activities in these studies are typically reported as a percentage of a maximum current (% I_{max}) generated by known substrate, such as L-glutamate, rather than as a percentage of the rate of the accumulation of a [³H]-substrate, such as [³H]-D-aspartate or [³H]-L-glutamate. This approach has also proven quite advantageous in resolving another key pharmacological issue problematic to standard

radiolabeled substrate-based competition assays; that is the differentiation of substrate and non-substrate inhibitors (also known as non-transportable inhibitors).³⁰ For example, the ability of an analog to competitively block the uptake of a radiolabeled substrate indicates that the analog is likely binding to the substrate site on the transporter protein, but provides little or no insight as to whether or not it can also be translocated intracellularly. Interestingly, recent structure-activity studies on EAAT2 suggest that the structural motifs regulating binding and substrate translocation are not necessarily one and the same.^{21,30}

EAAT pharmacology

As is the case with L-glutamate, all of the EAATs share the ability to utilize D-aspartate as a substrate. Further, the aspartate-based analog, β-threo-hydroxy-aspartate (THA) also competitively blocks uptake through all the EAATs, acting as an alternative substrate at EAAT1-4 and as a non-transportable inhibitor at EAAT5.^{2,3,22,32} The much used conformationally-restricted glutamate analog, L-trans-2,4-pyrrolidine dicarboxylate (L-trans-2,4-PDC) has a similar specificity, acting as a substrate of EAAT1-4

and as a non-substrate inhibitor of EAAT5.^{2,3,22,30,32} Among the 2-(carboxycyclopropyl)glycine (CCG) analogs that have been of such value in defining EAA receptor specificity, L-CCG-III has been shown to potently inhibit EAAT1,2 and 3.^{19,49} The THA analog β -*threo*-benzyloxy-aspartate (TBOA) has also generated considerable interest, as it is an effective non-substrate inhibitor of EAAT1 and one of the most potent non-substrate inhibitors of EAAT2 yet identified.⁴⁹

The greatest success in terms of selective action (and in the delineation of a pharmacophore model) has been achieved thus far with the EAAT2 subtype. Given its prevalence in the CNS and the high levels of activity present in the biochemical preparations used for so many years to define the fundamental structure-activity relationships of glutamate uptake (e.g. synaptosomes), it is not surprising that these studies self-selected compounds that acted at EAAT2. In addition to the classic inhibitor dihydrokainate (DHK), which is a selective non-transportable inhibitor of EAAT2, several more recently characterized analogs appear to preferentially act at this system relative to EAAT1 and EAAT3 (owing to their more recent isolation, fewer comparative studies have been conducted with EAAT4 and EAAT5). These compounds include: (2*S*,4*R*)-4-methylglutamate (4-MG),^{45,56} (\pm)-*threo*-3-methyl glutamate (3-TMG),^{20,56} L-*trans*-2,3-pyrrolidine dicarboxylate (L-*trans*-2,3-PDC),³⁰ L-*anti-endo*-3,4-methano-pyrrolidine-3,4-dicarboxylate (MPDC),¹⁴ L-CCG-IV,⁶¹ and (S)-2-amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl)propionic acid ((S)-TDPA).¹² Among these analogs, 4-TMG, 3-TMG, L-*trans*-2,3-PDC, and MPDC have been further characterized as non-substrate inhibitors of EAAT2. Within the context of describing these compounds, it is also important to use the terms "selective" and "preferential" with caution, as unforeseen activities at higher concentrations or as yet untested systems will undoubtedly come to light with time. For example, more recent studies with 3-TMG confirm its preferential action at EAAT2, compared to EAAT1 and EAAT3, but also demonstrate that it is a relatively good substrate of EAAT4.²⁰ Similarly, it is not uncommon for compounds that exhibit selectivity among the EAATs to also act as agonists or antagonists at EAA ionotropic or metabotropic receptors (e.g. DHK, L-*trans*-2,3-PDC, L-CCG-IV, (S)-TDPA). Although such activities do not preclude their value in defining the pharmacological and biochemical properties of the EAATs, it could limit their utility in more physiologically complex functional studies.

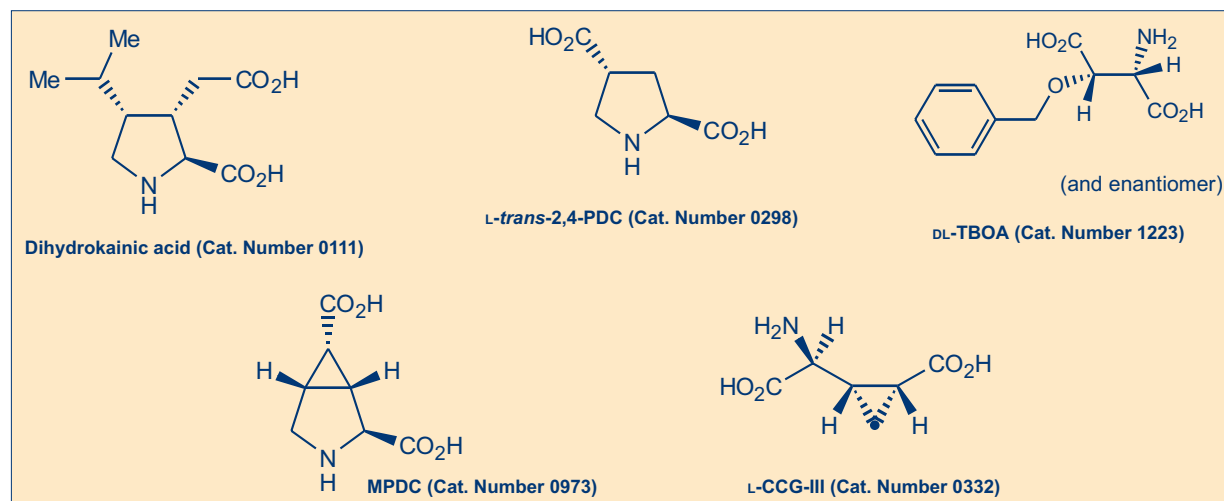
While progress in developing selective substrates and inhibitors for the transporters other than EAAT2 has not been as great, a number of interesting leads have surfaced that begin to differentiate each of the subtypes. For example, the ability to effectively use 4-MG and L-serine-O-sulfate (L-SOS) as substrates distinguishes EAAT1 from its counterparts.^{3,55} In contrast, THA and L-*trans*-2,4-PDC each appear to be substrates of all the EAATs except EAAT5, where the analogs act as potent non-transportable inhibitors.² Insight into developing novel compounds that selectively target EAAT3 and EAAT4, may be found in their relative sensitivities to inhibition by L-aspartate- β -hydroxamate and L- α -aminoadipate (L- α -AA), respectively.^{3,22}

Glutamate transport into synaptic vesicles

Prior to its depolarization-triggered, calcium-dependent release from neuron terminals, L-glutamate is transported into synaptic vesicles in an ATP-dependent manner by a specific uptake system.³⁷ The protein that mediates this uptake, referred to as VGLUT1, is unrelated to other neurotransmitter transporters and was originally characterized as a putative inorganic phosphate transporter.^{8,53} In contrast to the sodium-dependent EAATs, vesicular uptake is coupled to an electrochemical proton gradient generated by a vacuolar type ATPase (V-ATPase).^{33,37} Both a $\Delta\psi$ (inside positive) and a Δ pH (inside acidic) is formed by the V-ATPase in the presence of permeant chloride anions, although the extent to which each specifically contributes to the driving force remains undecided.^{23,33,51,60} Vesicular glutamate transport is inhibited by the anion channel blocker 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) in a manner that can be overcome with excess chloride ions, suggesting the presence of an additional ionic site at which uptake may be regulated.²⁷ In contrast to the high-affinity ($K_m = 5-50 \mu\text{M}$) EAAT systems, the K_m values determined for the uptake of glutamate into isolated synaptic vesicles are typically 1-2 mM.^{7,37}

In view of the detailed pharmacophore models emerging for the cellular EAAT proteins, it is clear that the pharmacological delineation of vesicular transport is still in its early stages. Indeed, identified inhibitors have varied tremendously in structural types, ranging from easily recognized glutamate mimics to molecules that are seemingly unrelated to the endogenous transmitter. For example, *erythro*-4-methyl-L-glutamate and 4-methylene-L-glutamate both attenuate the uptake of [³H]-L-glutamate into synaptic

Chemical structures of some EAAT inhibitors



(Bold text denotes compounds available from Tocris)

vesicles.⁵⁹ Interestingly, the vesicular system has also been shown to be competitively blocked by the glutamate metabotropic receptor agonist *trans*-ACPD and ionotropic receptor antagonist kynurenate.^{24,59} Further, the kynurenate analogs xanthurenate and 7-Cl-kynurenate are slightly more effective as inhibitors than kynurenate itself.⁷ Two of the most potent inhibitor classes identified to date are the ergots, (e.g. bromocriptine¹⁵) and the azo dyes, (e.g. Evans Blue, Chicago Sky Blue⁴⁶). These azo dyes are reported to inhibit the vesicular system with K_i values three to four orders of magnitude below the K_m for L-glutamate. In addition to these small molecules, recent studies have also identified a family of inhibitory protein factors (IPF $_{\alpha}$, IPF $_{\beta}$ and IPF $_{\gamma}$) that strongly bind to the transporter and block glutamate uptake into synaptic vesicles.⁴¹ While more systematic structure activity studies will be needed to develop a detailed pharmacophore model of the substrate site on vesicular glutamate transporters, the inhibitors described above should prove useful for both modulating transporter activity in functional studies and the design of more specific analogs.

Future directions

The library of compounds with which to characterize these various glutamate transport systems is multiplying rapidly, as is the number of studies employing selective substrates and inhibitors as probes of structure and function. Continued structure-activity analyses with conformationally constrained analogs will lead to increasingly detailed pharmacophore models of the substrate binding domains that should eventually be incorporated into the emerging topological models of transporter protein structure. Of particular value in these efforts will be the development of compounds, such as photoaffinity labels, that can effectively bind to the substrate site on the transporters and covalently modify the participating amino acids. From a functional perspective, a more complete library of selective inhibitors and substrates should also help to unravel the role of transport in excitatory signaling. Accumulating evidence suggests that the potential role of the EAATs in shaping the postsynaptic

excitatory signal needs to be evaluated in terms more complex than just simple uptake into surrounding cells. Emerging issues of interest include:

- i) understanding why transport inhibitors alter postsynaptic signaling in only certain subsets of glutamatergic synapses,^{6,17,28,47,54}
- ii) determining the significance of binding to, and translocation through, the transporters relative to the levels and timecourse of glutamate in the synaptic cleft^{5,10,17,35,39} and
- iii) elucidating the role of transport as a variable influencing the extent of synaptic spillover.^{9,18,48}

Another area of focus will be the use of novel substrates and inhibitors to probe the mechanism and physiological significance of the chloride conductances associated with EAAT activity.^{2,22,39,40,57,58} With respect to the vesicular glutamate transporter, more potent and selective inhibitors will be particularly useful in determining the role of the transporter in establishing the vesicular content of L-glutamate and, potentially, the amounts of synaptically released transmitter. Significantly, a recent study reported that treating synaptosomes with the inhibitory dye Rose Bengal resulted in a reduction in the depolarization-induced release of L-glutamate.³⁸ The potential to modulate excitatory activity in this manner will certainly attract marked attention in the near future.

Overall, the availability of an increasing number of pharmacological agents with which to selectively modulate the activity of the various glutamate transporters has been, and will continue to be, a critical step in elucidating their respective roles. While there is still much left to be accomplished, it is rewarding to see how these compounds have sparked a growing interest in glutamate transport and the recognition that it is an integral part of the excitatory amino acid system.

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EAA UPTAKE INHIBITORS AVAILABLE FROM TOCRIS

0271	<i>cis</i> -ACBD	Potent, selective L-glutamate uptake inhibitor
0332	L-CCG-III	Potent, competitive uptake inhibitor
0846	Chicago Sky Blue 6B	Potent inhibitor of L-glutamate uptake into synaptic vesicles
0237	7-Chlorokynurenic acid	Potent, competitive inhibitor of L-glutamate uptake
0134	(±)-Chlorpheg	L-homocysteate uptake inhibitor
0818	(2 <i>S</i> ,3 <i>R</i>)-Chlorpheg	L-homocysteate uptake inhibitor
0111	Dihydrokainic acid	EAAT2(GLT1)-selective non-transportable inhibitor of L-glutamate and L-aspartate uptake
0845	Evans Blue	Potent inhibitor of L-glutamate uptake into synaptic vesicles
0182	D(+)- <i>threo</i> -3-Hydroxyaspartic acid	Potent, competitive, transportable inhibitor
0183	L(-)- <i>threo</i> -3-Hydroxyaspartic acid	Potent, competitive, transportable EAAT1-4 inhibitor/non-transportable EAAT5 inhibitor
0811	(±)- <i>threo</i> -3-Methylglutamic acid	EAAT2 blocker
0973	MPDC	Potent inhibitor of L-glutamate uptake. Less activity as a substrate compared to its parent compound (0298)
0298	L- <i>trans</i> -2,4-PDC	Potent, competitive, transportable EAAT1-4 inhibitor/non-transportable EAAT5 inhibitor
1223	DL-TBOA	Potent, selective non-transportable inhibitor of EAATs

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