Pharmacology of Glutamate Transport in the CNS: Substrates and Inhibitors of Excitatory Amino Acid Transporters (EAATs) and the Glutamate/Cystine Exchanger System $\gamma_{\text{C}}$

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Abstract As the primary excitatory neurotransmitter in the mammalian CNS, L-glutamate participates not only in standard fast synaptic communication, but also contributes to higher order signal processing, as well as neuropathology. Given this variety of functional roles, interest has been growing as to how the extracellular concentrations of L-glutamate surrounding neurons are regulated by cellular transporter proteins. This review focuses on two prominent systems, each of which appears capable of influencing both the signaling and pathological actions of L-glutamate within the CNS: the sodium-dependent excitatory amino acid transporters (EAATs) and the glutamate/cystine exchanger, system $\gamma_{\text{C}}$ ($S\gamma_{\text{C}}$). While the family of EAAT subtypes limit access to glutamate receptors by rapidly and efficiently sequestering L-glutamate in neurons and glia, $S\gamma_{\text{C}}$ provides a route for the export of glutamate from cells into the extracellular environment. The primary intent of this work is to provide an overview of the inhibitors and substrates that have been developed to delineate the pharmacological specificity of these transport systems, as well as be exploited as probes with which to selectively investigate function. Particular
attention is paid to the development of small molecule templates that mimic the structural properties of the endogenous substrates, L-glutamate, L-aspartate and L-cystine and how strategic control of functional group position and/or the introduction of lipophilic R-groups can impact multiple aspects of the transport process, including: subtype selectivity, inhibitory potency, and substrate activity.

**Keywords** Aspartate · Aspartylamide · Benzyloxyaspartate · Pyrrolidine-dicarboxylate · Uptake

**Abbreviations**

| Abbreviation | Structure/Description |
|--------------|-----------------------|
| #24          | 3-{[3′-Trifluoromethyl-2-methyl-1,1′-biphenyl-4-yl]carbonyl}amino-L-alanine |
| 4-MG         | (2S,4R)-4-Methyl glutamate |
| 2,4-MPDC     | 2,4-Methanopyrrolidine-2,4-dicarboxylate |
| (2S,3R)-3-Me-L-trans-2,3-PDC | (2S,3R)-3-Methyl-L-trans-2,3-pyrrolidine dicarboxylate |
| (2S,4R)-4-Me-L-trans-2,4-PDC | (2S,4R)-4-Methyl-L-trans-2,4-pyrrolidine dicarboxylate |
| (4S,2′S)-APOC | (4S,2′S)-2-[2′-Aminopropionate]-4,5-dihydro-oxazole-4-carboxylic acid |
| (4S,5S)-POAD | (4S,5S)-2-Phenyl-4,5-dihydro-oxazole-4,5-dicarboxylate |
| AHTP         | (R,S)-2-Amino-3-(1-hydroxy-1,2,3-triazol-5-yl)propionate |
| AMPA         | α-Amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid |
| CNB-TBOA     | (2S,3S)-3-[3-(4-Cyanobenzyloxy)benzoyloxy]aspartate |
| DHK          | Dihydrokainate |
| EPSC         | Excitatory postsynaptic current |
| ETB-TBOA     | (2S,3S)-3-3-[4-Ethylbenzoyl]benzyl]oxy]aspartate |
| KA           | Kainic acid |
| L-anti-endo-3,4-MPDC | L-anti-endo-3,4-Methanopyrrolidine dicarboxylate |
| L-AP4        | L(+)2-Amino-4-phosphonobutyric acid |
| L-β-THA      | L-β-threo-Hydroxy-aspartate |
| L-SOS        | L-Serine-O-sulphate |
| L-TBOA       | L-threo-Benzoyl aspartate |
| L-trans-2,3-PDC | L-trans-2,3-Pyrrolidine dicarboxylate |
| L-trans-2,4-PDC | L-trans-2,4-Pyrrolidine dicarboxylate |
| NBI 59159    | N-4-(9H-Fluoren-2-yl)-L-asparagine |
| NMDA         | N-Methyl-D-aspartate |
| PMB-TBOA     | (2S,3S)-3-[3-(4-Methoxybenzyloxy)benzyl]oxy]aspartate |
| (S)-4-CPG    | (S)-4-Carboxy-phenylglycine |
| T3MG         | threo-3-Methylglutamate |
| TDPA         | S-2-Amino-3-(3-hydroxy-1,2,5-thiadiazol-5-yl)propionic acid |
| TFB-TBOA     | (2S,3S)-3-[3-(4-Trifluoromethyl)-benzoyl]amo]benzoyloxy]aspartate |
| WAY-855      | 3-Amino-tricyclo[2.2.1.0[2.6]heptane-1,3-dicarboxylate |
| WAY 212922   | N[4]-[7-(Trifluoromethyl)-9H-fluoren-2-yl]-L-asparate |
1 Introduction

As our understanding of the physiological and pathological significance of glutamate-mediated neurotransmission has increased over the past 40 years, so too has our interest in the development of the excitatory amino acid (EAA) analogues that can be used to study the structure, function, and therapeutic relevance of the participating proteins. Now recognized as the primary excitatory neurotransmitter in the mammalian CNS, this dicarboxylic amino acid participates not only in standard fast synaptic communication, but also contributes to the cellular signaling that underlies higher order processes such as development, synaptic plasticity, learning and memory [1].

To a large extent, the ability of L-glutamate (L-Glu) to participate in such a wide variety of functions is a result of the large diversity of EAA receptor subtypes and splice variants available for signal mediation. These receptors include those directly associated with ion channels (ionotropic receptors, iGluRs), such as NMDA, KA and AMPA receptors, as well as those coupled to second messenger systems (metabotropic receptors, mGluRs). The iGluRs have garnered significant attention from both mechanistic and therapeutic perspectives, because the over-activation of these receptors and subsequent influx of excessive levels of ions (e.g., Ca\(^{2+}\), Na\(^{+}\)) is now accepted as the fundamental pathological pathway (i.e. excitotoxicity) underlying neuronal injury in both acute insults (e.g., stroke, trauma, hypoglycemia) and chronic neurological and neuropsychiatric disorders (e.g., amyotrophic lateral sclerosis, epilepsy, schizophrenia, Alzheimer’s disease, Huntington’s disease) [2–8]. The mGluR’s, on the other hand, are more typically associated with the modulation of excitatory transmission through a variety of G-protein coupled signal transduction pathways [9, 10]. In addition to neurodegenerative pathways, interest in these receptors has been linked to a number of disorders where a therapeutic benefit may be found in establishing new balance points in excitatory signaling through regulatory mechanisms, including epilepsy, anxiety and pain management [9, 11, 12]. Not surprisingly, much of the progress made in understanding the pharmacology and physiological roles of EAA receptors have been dependent upon strong medicinal chemistry efforts that have yielded growing libraries of receptor specific agonists and antagonists.

More recently, attention has focused on another family of proteins that are also becoming accepted as integral to excitatory signaling i.e., the transport systems that regulate the extracellular and intracellular concentrations

WAY 213394  \(N[4]-\left(2^\prime\right.\text{-Methyl-1,1'\text{-biphenyl-4-yl}}\right)\text{-L-asparagine}\)
WAY 213613  \(N4-[4-(2\text{-Bromo-4,5-difluorophenoxy})\text{phenyl}]\text{-L-asparagine}\)
of glutamate in the CNS. Given the ability of the transporters to efficiently control the movement of glutamate into and out of cellular and subcellular compartments, these proteins have the potential to influence the amount and time-course of the excitatory transmitter that reaches synaptic and extrasynaptic receptors, thereby influencing both physiological signaling and pathological injury. Not surprisingly, this has also created a need for additional EAA analogues with which to selectively characterize these transport proteins. The present review focuses on two cellular transport systems that appear to have a significant impact on excitatory transmission and/or excitotoxicity: the sodium-dependent excitatory amino acid transporters (EAATs) and the glutamate/cystine exchanger, system \( x_c^- \) (S\( x_c^- \)). The EAATs have long been recognized for the ability to efficiently sequester L-Glu in neurons and glia, thereby providing a route to clear this transmitter from the extracellular space and regulate its access to EAA receptors. \( S_x^- \), on the other hand, functions as an obligate exchanger that typically couples the import of L-cystine (L-Cys\(_2\)) with the export of L-Glu. While the uptake of L-Cys\(_2\) has been the more common focus of studies on \( S_x^- \), recent evidence suggests that the resulting export of L-Glu may represent a non-synaptic source of this excitatory transmitter that may also contribute to both physiological and pathological signaling. This review is intended to complement other works examining the structure, regulation, and physiology of these transporters [13–19], by placing an emphasis on the small molecules that have been developed to delineate the pharmacological specificity of these two transport systems, as well as be exploited as probes with which to assess their potential contributions to CNS signaling and pathology.

2 Excitatory Amino Acid Transporters (EAATs)

Among the uptake systems addressed in this review the excitatory amino acid transporters (EAATs) have been studied for the longest period of time and, not surprisingly, are the most thoroughly characterized. Interest in glutamate transport initially arose within the context of establishing that this dicarboxylic acid was indeed an excitatory transmitter, as it provided a mechanism to meet the requisite criteria of transmitter inactivation in the absence of a synaptically localized degradative enzyme akin to acetylcholinesterase. The identification of the high affinity glutamate uptake systems was also key to unraveling the complexities of its metabolic compartmentalization in the brain and the recognition of the glutamine cycle, in which synaptically released glutamate is rapidly transported into astrocytes and converted to glutamine by glutamine synthetase [20]. In turn, the glutamine is shuttled back to the presynaptic terminal, where it is reconverted into glutamate by glutaminase for repackaging into synaptic vesicles. The significance of
this pathway, and particularly the maintenance of low extracellular levels of glutamate, became increasingly evident as the pathological properties of L-Glu were delineated. Referred to as excitotoxicity, the over activation of ionotropic EAA receptors by excitatory agonists produces an excessive influx of ions (particularly Na\(^+\) and Ca\(^{2+}\)) that triggers a number of pathological pathways \[6, 21–23\]. Glutamate-mediated neuronal injury has become increasingly recognized as a fundamental mechanism of CNS pathology that is now linked to acute insults (e.g., stroke, head trauma, spinal cord injury) as well as chronic neurodegenerative diseases (e.g., amyotrophic lateral sclerosis, Alzheimer’s, Parkinson’s and Huntington’s disease \[5, 24–27\].

2.1 Properties

Initially characterized on a basis of activity, the transport system was distinguished by its sodium dependency and high affinity \((K_m \approx 1–5 \, \mu M)\) for L-Glu \[28\]. To date, five isoforms of the EAATs have been isolated and characterized. The first three were identified almost simultaneously: GLAST (EAAT 1) \[29\] and GLT1 (EAAT 2) \[30\], which are principally considered glial transporters, from rat brain and EAAC1 (EAAT 3) \[31\], a neuronal transporter, from rabbit intestine. In addition to the subsequent identification of the three homologous transporters from human brain \[32\], EAAT 4 and EAAT 5 were isolated from cDNA libraries of human cerebellum and human retina, respectively \[33, 34\]. On the basis of substrate specificity, EAATs 1–5 are considered as members of System \(X^-\text{AG}\), while molecular analysis reveals that the EAATs are members of a novel gene family [Human Genome Organization solute carrier family (SLC1)] that also includes the sodium-dependent neutral amino acid transporters ASCT1 and ASCT2 \[35, 36\]. EAAT 1–3 have been reported to be present outside the nervous system, while EAAT 4 is believed to be restricted to cerebellar and EAAT 5 to the retina \[13, 37\].

The EAATs are thought to function using an “alternate access” gating mechanism in which the substrate initially interacts with an outwardly facing binding domain \[38, 39\]. The binding of the substrate and requisite ions then produces a conformational change that orients the protein such that the substrate now has access to the intracellular compartment. In the instance of the EAATs, the transport of one molecule of L-Glu is coupled to the inward movement of 3 Na\(^+\) ions and a H\(^+\) \[40\]. Interestingly, recent crystallographic data (see below) suggest that a minimum of two sodium ions are needed for binding, while the third may be more closely linked to the transition/translocation step \[39\]. The reorientation that allows the binding domain to again be accessed extracellularly is coupled to the export of one K\(^+\) ion. The Na\(^+\) and K\(^+\) gradients generated in CNS cells allows L-Glu to be concentrated intracellularly more than 5 orders of magnitude and provides a way to maintain L-Glu extracellular concentrations at a level that do not induce excitotoxic in-
Further, the stoichiometric ratio of substrate and its co-transported ions also makes the uptake of L-Glu electrogenic [41]. Advantageously, this property enables the transporter’s activity, pharmacology and biophysics to be investigated using electrophysiological recording techniques. Using such approaches, the transporters have also been found to exhibit ion conductances that are stoichiometrically independent of uptake and more consistent with channel-like properties [33, 34, 42].

The publication of a crystal structure of an archaeal glutamate transporter homologue from *Pyrococcus horikoshii* (GltPh) by Gouaux and colleagues in 2004 was a major turning point in understanding the molecular structure of the EAATs [43]. While a number of previous studies supported the conclusion that the EAATs exist as homomultimers, the number of proposed noncovalently associated subunits ranged from dimers to pentamers [44–46]. In the instance of GltPh, the transporter is composed of three subunits that assemble into a bowl-like configuration with its solvent-filled basin facing the extracellular surface. Each subunit is composed of eight α-helical transmembrane segments and two helical hairpin loops. Subsequent studies on GltPh, have yielded more clearly resolved binding sites for the substrate, as well as two of three requisite sodium ions needed for uptake [39]. Access to these sites appears to be gated by one of the two hairpin loops, HP 2. In the mechanistic model emerging from these studies, the binding of substrate and sodium prompts the movement and stabilization of the HP 2 loop in a closed configuration. Although not yet demonstrated, the HP 1 loop may play an analogous role in the subsequent gating of the substrate and access of ions to the intracellular compartment. Interestingly, when GltPh was crystallized with L-TBOA, rather than L-aspartate, the aryl group of this competitive inhibitor appeared to interact with the HP 2 and prevent this presumed gate from closing. Such an effect would explain the action of TBOA, as well as a number of other analogues discussed in the pharmacology section, as a non-substrate inhibitor; i.e., a ligand that binds to the substrate site but cannot be translocated across the membrane. Having such structural models in hand provides an important new contextual framework with which to evaluate our understanding of the pharmacological specificity of the EAATs that emerged from traditional SAR studies.

### 2.2 Pharmacology

Much of the pharmacological characterization of the EAATs have revolved around traditional competition assays, where EAA analogues are assayed for the ability to reduce the uptake of a radiolabeled substrate, typically $^3$H-L-Glu, $^3$H-L-aspartate or $^3$H-D-aspartate. The latter of these three has been extensively employed because it affords the advantage of being metabolically inert. Early studies relied on a variety of CNS preparations, such as synapto-
sommes tissue slices, or primary cell cultures, while current experiments more often employ cells transfected to selectively express one of the five isolated subtypes. *Xenopus* oocytes have played an especially important role in this regard, not only as an expression system for cloning the subtypes, but also because of the ease with which electrophysiological techniques can be used to exploit the electrogenic character of EAAT-mediated uptake and quantify substrate-induced currents in these cells [47]. This approach has allowed inhibitors previously identified in radiolabeled flux studies to be further differentiated into alternative substrates or non-substrate inhibitors [48]. Indeed, it is now common to find substrates characterized on a basis of maximum current produced ($I_{\text{max}}$), rather than the traditional $V_{\text{max}}$ value generated in radiolabel flux experiments.

Initial studies delineating the pharmacology of EAAT-mediated uptake were carried out prior to the isolation of the individual subtypes and therefore relied upon CNS preparations containing a mixture of isoforms. Nonetheless, a number of fundamental structure–activity relationships emerged from these experiments that may generalize to ligands capable of binding to most or all of the EAATs [28, 49, 50]. These include being an $\alpha$-amino acid with a second acidic/charged group separated from the $\alpha$-carboxylate by 2–4 carbon atoms. The uptake systems exhibit an enantioselectivity that is observed with the longer length ligands, such as L-Glu, but diminishes with the shorter length analogues; e.g., D-aspartate and D-cysteate are active not only as inhibitors, but also as substrates. While little if any variability is tolerated with respect to the $\alpha$-carboxyl group, the distal carboxylate can be replaced with sulfinic and sulfonic, but not phosphate-containing, charged groups. Some limited modification can also be made to the distal carboxylate without loss of inhibitory activity, such as derivatization to a hydroxamate. Additional steric bulk appended directly on the carbon backbone also appears to be tolerated in the transporter binding site, as illustrated by the inhibitory activity of threo-OH-aspartate, 3-methyl-, and 4-methyl-glutamate [51]. In particular the hydroxy-aspartate provided a template for the development of the very potent TBOA library of inhibitors [52].

Further progress in the development of more potent inhibitors, as well as the first major inroads into subtype-selective ligands, emerged through the development and application of conformationally constrained analogues [53]. In these compounds, ring systems and/or steric bulk are introduced so as to limit the conformational flexibility of the compounds. As a result of this increased rigidity, the compounds mimic fewer of the conformations attainable by glutamate and, consequently, interact with a more limited range of glutamate binding sites. In addition to conformational bias, appending alkyl or aryl groups to the carbon backbone of substrates such as L-Glu or L-aspartate also serve to facilitate interactions between the ligands and lipophilic protein domains adjacent to the substrate binding site. As will be discussed below, such side group interactions may be key to enhancing the potency and/or
subtype specificity of the inhibitors, as well as a determining factor in the differentiation of alternative substrates from non-substrate inhibitors. Another benefit of the conformational restrictions, particularly evident in compounds in which a glutamate or aspartate mimic is embedded in a ring system, is that the positions of the functional groups can be accurately mapped in 3D space and incorporated into SAR-based pharmacophore models [54–56]. Examples of analogues designed using both acyclic and cyclic templates can be found in the groups of inhibitors discussed below.

2.2.1 Substituted Aspartate Analogues

Long recognized as an alternative substrate, L-aspartate (Fig. 1a) has proven to be of particular value as a “backbone” template in the design of EAAT inhibitors. Indeed, two of the most potent families of blockers identified to date can be traced back to two aspartate analogues characterized in some of the earliest SAR work focused on glutamate uptake: β-threo-hydroxyaspartate (β-THA) and L-aspartate-β-hydroxamate (Fig. 1b,c) [50]. β-THA was shown to be an effective uptake blocker in tissue slices, synaptosomes and cultured astrocytes, while more recent studies with the cloned EAATs demonstrate that it acts as a competitive inhibitor at all five EAAT subtypes [47, 57]. Kinetic studies yielded $K_i$ values that range from a low of about 1 µM (EAAT 4 and 5) to a high of about 40 µM at EAAT 3. Interestingly, β-THA also exhibits a wide range of activities with respect to its ability to serve as a substrate and be translocated across the plasma membrane. Thus, based on substrate-induced currents (i.e., $I_{max}$), β-THA is similar to L-Glu at EAATs 3 and 4,

![Fig. 1 β-Substituted aspartate analogues](image-url)
about 30–70% as active as L-Glu at EAATs 1 and 2, and a non-substrate inhibitor of EAAT 5.

A significant advance in the development of much more potent inhibitors came from Shimamoto and colleagues who discovered that the hydroxy group of β-THA could serve as a linkage point for attachment of a variety of side chains. These compounds were initially synthesized with ester linkages and included the acetoxy, propionyloxy, benzoyloxy, and (1-, 2-napthyl) oxy derivatives [52]. While these compounds proved to be effective competitive inhibitors when tested at the EAATs (most often EAAT 1 or 2), their use was limited because of chemical instability attributable to ester cleavage or acyl migration. Importantly, however, these results led to the development of comparable analogues that incorporated an ether linkage. Especially noteworthy was D,L-TBOA (Fig. 1d), which was identified at the time as one of the most potent EAAT 2 inhibitors yet recognized, exhibiting $K_i$ values in the low to sub-$\mu$M range, depending upon the assay employed [58]. Further characterization revealed that: (i) more inhibitory activity resided with the L-enantiomer, (ii) TBOA competitively blocked all five EAATs, and (iii) in each instance TBOA was acting as a non-substrate inhibitor. Continued work by the Shimamoto group has produced a growing library of TBOA derivatives that included modifications to the benzyl moiety, as well as its replacement with a variety of aryl groups [59]. In addition to demonstrating that the presumed lipophilic pocket with which these side groups were binding could accommodate substantial steric bulk, some of the analogues were found to be significantly more potent than the parent compound. Thus, (2S,3S)-3-[3-(4-methoxy-benzoylamino)benzyloxy]aspartate (PMB-TBOA) (Fig. 2a), (2S,3S)-3-[3-[4-(trifluoromethoxy)benzoylamino]benzyloxy]aspartate CF$_3$O-Bza-TBOA and

![Fig. 2 L-threo-Benzylloxy aspartate analogues](image-url)
(2S,3S)-3-{3-[4-(trifluoromethyl)benzoylamino]benzyloxy}aspartate (TFB-TBOA) (Fig. 2b) each exhibited IC₅₀ values in the 1–10 nM range. Equally significant, these modifications also increased the selectivity of some of the analogues. For example, PMB-TBOA, (2S,3S)-3-[3-(4-cyanobenzoylamino)benzyloxy]aspartate (CNB-TBOA) (Fig. 2c) and (2S,3S)-3-[3-(4-flourobenzoylamino)benzyloxy]aspartate (F-BzA-TBOA) are each 20 to 40-fold more potent at EAAT 2 relative to EAAT 3. Most recently a tritiated derivative of (2S,3S)-3-{3-[4-ethylbenzoylamino]benzyloxy}aspartate (ETB-TBOA) (Fig. 2d) was synthesized and used as a radioligand to selectively investigate the binding properties of the EAATs [60]. This compound exhibited Kᵋ values of 10 to 30 nM for EAATs 1, 2, 4 and 5, and 320 nM for EAAT 3. Beyond their value in distribution studies, such radioligands can serve as standards to cross-correlate SAR data that is strictly based upon ligand binding, much in the same way as has been done in studies of monoamine transporters [61, 62].

Esslinger and coworkers recently prepared the β-substituted aspartate analogue in which the benzyl group is attached to the carbon backbone via a methylene bond, rather than the ether linkage used in TBOA [54]. Significantly, l-β-benzyl-aspartate (Fig. 1e) proved to be one of the first competitive inhibitors identified that preferentially blocked EAAT 3. The threo diastereoisomer, which was more potent than the erythro, yielded a Kᵋ value in C17 cells expressing EAAT 3 (0.8 µM) that was about 10-fold lower than found with either EAAT 1 or 2. Electrophysiological recording in oocytes expressing EAAT 3 confirmed that l-β-threo-benzyl-aspartate is a non-substrate inhibitor. Computational modeling suggested that while the position of the carboxylate and amino groups are very similar, the differential activity at EAAT 3 may reside in the orientation of the benzyl group and/or the presence of the ether oxygen and how these specifically interact with the binding domains of the individual EAATs.

2.2.2 Substituted Glutamate Analogues

While it has not proved quite as fruitful in terms of producing highly potent or specific inhibitors, similar modifications to the carbon backbone of L-Glu (Fig. 3a) has yielded a number of compounds that exhibit intriguing activities at the EAATs. Initial studies, many of which were carried out by Vandenberg and colleagues, focused on the addition of methyl or hydroxyl groups to the 3 and 4 positions of glutamate. In this manner, the (±)-erythro diastereoisomer of 3-methyl-glutamate was shown to exhibit little or no activity at either EAAT 1 or 2, while (±)-threo-3-methyl-glutamate (Fig. 3b) was found to be inactive at EAAT 1, yet act as a non-substrate inhibitor of EAAT 2 (Kᵋ ≈ 20 µM; [51]). In the instance of substitutions at the 4-position, both hydroxy and methyl groups are tolerated, but exhibit stereo-specific differences [51, 63]. Thus, (2S,4S)-4-hydroxy-glutamate is an alternative substrate
of both EAAT 1 ($K_m \approx 61 \mu M$) and EAAT 2 ($K_m \approx 48 \mu M$), while the 2S,4R isomer appears inactive. In contrast, (2S,4S)-4-methylglutamate is inactive at EAATs 1 and 2, while the 2S,4R isomer (Fig. 3c) is an alternative substrate of EAAT 1 ($K_m \approx 54 \mu M$) and a non-substrate inhibitor of EAAT 2 ($K_i \approx 3 \mu M$). When substitutions at the 4-position of L-Glu were markedly expanded to include a variety of alkyl groups, the resulting SAR data was consistent with TBOA-based analogues with respect to the conclusion that the binding sites of EAATs 1–3 could accommodate considerable steric bulk, although the modifications did not yield a comparable increase in inhibitory potency. With the exception of the ability of EAAT 1 to use (2S,4R)-methylglutamate as a substrate, all of the alkyl-substituted analogues proved to be non-substrate inhibitors at all three transporters. (2S,4R)-4-Benzyl-glutamate was also of particular note because it preferentially inhibited EAAT 1, compared to either EAAT 2 or 3 [63].

### 2.2.3 Amide Derivatives of Aspartate and Diaminopropionate

Another valuable series of EAAT inhibitors has emerged from collaborative work of Dunlop, Foster, Butera and colleagues, using L-aspartate and diaminopropionate as structural templates [64, 65]. Rather than TBOA-like molecules, in which additions were made to the C3 position, this new series of analogues employed amide linkages to mimic the distal carboxylate group and as a point of attachment for a wide variety of aryl groups. This modification is particularly interesting as it suggests that the distal carboxylate, unlike its α counterpart, can be partially “masked”. While the ability of such a modification to be tolerated in the binding site was suggested by early studies examining the activity of aspartate and glutamate hydroxamates, this library of analogues is particularly interesting because it contains inhibitors that both exhibit $IC_{50}$ values in the sub-μM range and display increased subtype selectivity. For example, WAY-213394 ($IC_{50} \approx 0.1 \mu M$), WAY-2129222 and WAY-0213613 (Fig. 4a–c) are reported to be between 20 to 100-fold more potent as inhibitors of EAAT 2 ($IC_{50} \approx 0.1 \mu M$), then either EAAT 1 or 3. Equally interesting are analogues such as NBI-59159 ($IC_{50} \approx 0.1 \mu M$) and 3-{{3′-trifluoromethyl-2-methyl-1,1′-biphenyl-4-yl)carbonyl}amino}-L-alanine cited by Greenfield and co-workers as #24 [64]
Fig. 4 Amide derivatives of aspartate and diaminopropionate (IC$_{50} \approx 9$ µM) (Fig. 4d,e) that are among the first to show preferential activity at EAAT 3.

### 2.2.4 Carboxy-cyclopropyl and Cyclobutyl Analogues

As an alternative to appending functional groups directly to a glutamate or aspartate template, a number of inhibitor collections have exploited ring systems to bias, and often at times limit, the spatial configuration of the required carboxylic and amino function of the excitatory amino acids. The carboxy-cyclopropylglycines have proven quite advantageous in this respect, as this parent structure has led to pharmacological probes of not only transporters, but also ionotropic and metabotropic receptors [66, 67]. Three of the isomers, CCG-II, III and IV, effectively inhibited the transport of L-Glu, although these initial studies were primarily carried out in heterogeneous CNS preparations (e.g., synaptosomes, glial plasmalemmal vesicles), rather than with isolated EAAT subtypes [68]. In later studies employing expressed EAATs, both CCG-III and CCG-IV (Fig. 5a,b) were shown to inhibit EAAT 2 with IC$_{50}$ values of 0.3 and 1 µM respectively, while CCG-II produced no inhibitory activity when tested at 10 µM [69]. Subsequent studies reported that CCG-III blocked EAAT 1 and EAAT 3, as well as EAAT 2 (although the $K_i$ values were somewhat higher, e.g., 2.5–10 µM), but that CCG-IV was considerably less potent at EAATs 1–3 ($K_i$ or IC$_{50}$ values ranging from 170 to 900 µM) [58, 70].
A related series of L-2-(2-carboxycyclobutyl)glycines (CBG’s) was recently prepared using an aspartate aminotransferase in a chemo-enzymatic synthesis and assessed for EAAT activity with a FLIPR-based membrane potential assay [71, 72]. While none of the CBG isomers tested proved to be exceptionally potent inhibitors, L-CBG-I was found to be a substrate for EAAT 1–3, albeit with \( K_m \) values 10 to 20-fold greater than for L-Glu. In contrast, L-CBG-II (Fig. 5c) inhibits all three EAATs, acting as an alternative substrate of EAAT 1 (\( K_m = 96 \mu M \)) and a non-substrate inhibitor of EAATs 2 and 3 (\( K_m = 22 \) and 49 \( \mu M \), respectively). A marked subtype selectivity (up to about 30-fold) was observed with L-CBG-III and -IV (Fig. 5d,e), which are non-substrate inhibitors of EAAT 2 and 3, but exhibit markedly less activity at EAAT 1. Of all the CBG’s characterized, CBG-IV was reported to be the most potent, yielding \( K_i \) values of 7 and 10 \( \mu M \) at EAATs 2 and 3, respectively [71].

### 2.2.5 Pyrrolidine Dicarboxylate (PDC) Analogues

One of the earliest cyclic analogues of glutamate to garner attention as an uptake inhibitor was dihyrokainate (DHK) (Fig. 6a), the reduced form of the classic iGluR agonist kainate [73]. While first characterized as a transport blocker in heterogeneous CNS preparations, interest in DHK has remained, as studies with the isolated EAAT subtypes identified it as a selective inhibitor of EAAT 2 [32]. The same five-membered pyrrolidine ring present in DHK is also found in a series of analogues in which the distal carboxylate group is directly attached to the pyrrolidine ring rather than via a methylene linkage. In the first of this series, carboxylate groups were placed at the 2 and 4 positions to mimic glutamate. Of the four diastereoisomers, L-trans-2,4-pyrrolidine dicarboxylate (L-trans-2,4-PDC) (Fig. 6b) was found to be the most potent...
Fig. 6 Pyrrolidine dicarboxylate (PDC) analogues

inhibitor of synaptosomal L-Glu transport \( (K_i \approx 2–5 \, \mu M) \) [74]. Significantly, L-trans-2,4-PDC also exhibited little if any activity at EAA ionotropic receptors. When later examined against the individual EAAT subtypes, L-trans-2,4-PDC was shown to: (i) inhibit all five subtypes, (ii) act as an alternative substrate of EAATs 1–4 \( (K_m \approx 28, 7, 27 \text{ and } 3 \, \mu M, \text{ respectively}) \) and (iii) be a non-substrate inhibitor of EAAT 5 \( (EC_{50} \approx 6 \, \mu M) \) [32–34]. Repositioning the distal carboxylate mimic from the 4 to the 3 position on the pyrrolidine ring yielded a more “aspartate-like” compound that was void of substrate activity at EAAT 2, yet acted at this subtype as a non-substrate inhibitor \( (K_i \approx 10 \, \mu M) \) with an enhanced selectivity \( (\approx 10 \text{ to } 20 \text{ fold}) \), compared to EAATs 1–4 ([48] and M.P. Kavanaugh, personal communication). With a functional group configuration more similar to aspartate, it was not surprising that L-trans-2,3-PDC (Fig. 6c), unlike L-trans-2,4-PDC, is a potent NMDA agonist and excitotoxin [75].

Further restricting the PDC template through the addition of methylene bridges has also demonstrated that subtle structural changes can produce compounds with very divergent properties at the EAATs, particularly as related to the ability of analogues to serve as alternative substrates. For example, L-anti-endo-3,4-MPDC (Fig. 6d) is a non-substrate inhibitor of EAAT 2, exhibiting \( K_i \) values of \( \approx 5 \, \mu M \) synaptosomes (in which the EAAT 2 subtype is predominant) and \( \approx 9 \, \mu M \) in oocytes expressing EAAT 2. The closely related 2,4-methanopyrrolidine-2,4-dicarboxylate (2,4-MPDC) (Fig. 6e) also binds and competitively inhibits synaptosomal uptake of \(^3\)H-D-aspartate \( (K_i \approx 7 \, \mu M) \), yet appears to be translocated quite effectively by EAAT 2, as it yielded an \( I_{\text{max}} \) value that is actually greater \( (115\%, \, K_m \approx 45 \, \mu M) \) than that produced by the endogenous substrate L-Glu [48]. Interestingly, these results suggest that conformational restriction does not
necessarily attenuate substrate suitability. Analogous changes in activity can also be produced by introducing methyl groups into the PDC template. Thus, both L-\textit{trans}-2,4-PDC and (2S,4R)-4-methyl-2,4-PDC (Fig. 6f) act as competitive inhibitors of EAAT 2-mediated uptake, ($K_i = 2-3$ $\mu$M in synaptosomes), although the presence of the methyl group essentially converts L-\textit{trans}-2,4-PDC from a substrate to a non-substrate inhibitor ([55], also confirmed in oocytes expressing EAAT 2 by M.P. Kavanaugh). Taken together, these SAR studies highlight that the value of the PDC’s rests not as much with their potency as inhibitors, but with their constrained structures that allow requisite functional group positions to be identified and exploited in the generation of pharmacophore models for both binding and translocation.

2.2.6 Heterocyclic and Carbocyclic Analogues

A number of other alternative ring systems have also been employed in the development of EAAT inhibitors, several of which were initially used as probes of EAA receptor pharmacology. For example, the library of azole-containing heterocyclic analogues used in the development of AMPA receptor agonists and antagonists by Krogsgaard-Larsen, Stensbol, Madsen, Brauner-Osborne and colleagues have also yielded compounds that exhibit activity at the EAATs [76, 77]. In particular, (R,S)-2-amino-3-(1-hydroxy-1,2,3-triazol-5-yl)propionate (AHTP) (Fig. 7a) inhibited $^3$H-D-aspartate uptake into Cos-7 cells expressing EAAT 1 and EAAT 2 with IC$_{50}$ values of about 100 and 300 $\mu$M, respectively. The compound appeared inactive at EAAT 3. This activity is notable not so much in terms of potency, but because this triazole derivative is one of the few compounds that preferentially acts at EAAT 1, rather than EAAT 2. In contrast, S-2-amino-3-(1-hydroxy-1,2,5-thiadiazol-4-yl)propionic acid (TDPA) (Fig. 7b), a related analogue in which the central nitrogen of the triazole group was replaced with sulfur, proved to be a selective inhibitor of EAAT 2 ($K_i \approx 100$ $\mu$M) [77]. Comparisons between these compounds and related AMPA analogues also led to the supposition that AMPA itself does not bind to the EAATs because its 5-methyl substituent is not tolerated within the binding site.

A similar series of analogues incorporating oxazole and oxazoline ring systems were developed by Campiani and coworkers and also shown to act as EAAT inhibitors [78, 79]. While also nitrogen-containing heterocyclics, these compounds, like the PDC’s, possess carboxylate groups directly attached to the five-membered ring. Interestingly, when tested against EAAT 3 (EAAC 1) expressed in HEK-293 cells, (4S,2'S)-2-[2'-aminopropionate]-4,5-dihydro-oxazole-4-carboxylic acid ((4S,2'S)-APOC) (Fig. 7c) was found not only to bind, but to act as a substrate of the transporter, exhibiting an $I_{\text{max}}$ comparable to L-Glu and a $K_m$ value of about 30 $\mu$M. Like AHTP and TDPA, the presumed $\alpha$-amino and $\alpha$-carboxylate mimics of L-Glu are incorporated
Fig. 7 Heterocyclic and carbocyclic EAAT inhibitors

into APOC via a 2-amino-3-propionate group. If the linkage to the ring is increased by another methylene group, it results in a marked loss of activity. Interestingly, it appears as if the nitrogen of the oxazole ring can also function as an amino group mimic, similar to the PDC’s, as \((4S,5S)-2\)-phenyl-4,5-dihydro-oxazole-4,5-dicarboxylate (4\(S\),5\(S\)-POAD) (Fig. 7d) inhibited EAAT 3 with a \(K_i\) value of 14 \(\mu M\) [78]. In contrast to 4\(S\),2\(S\)-APOC, however, 4\(S\),5\(S\)-POAD acts as a non-substrate inhibitor.

Rather than a heterocyclic template, Dunlop and coworkers have also developed a tricycloheptane-based analogue, 3-amino-tricyclo[2.2.1.0\[2.6\]]heptane-1,3-dicarboxylate (WAY-855) (Fig. 7e), that is somewhat akin to the PDC analogues in its use of conformational restriction, with the exception that the \(\alpha\)-amino acid group mimic is not embedded within the ring [80]. WAY-855 has attracted considerable attention because it displays a very strong preference for EAAT 2, compared to either EAAT 1 or 3. When tested in oocytes expressing EAATs 1–3, the analogue was reported to be a non-substrate inhibitor of all three subtypes that was 50-fold more potent at EAAT 2, where it exhibited an IC\(_{50}\) of about 1 \(\mu M\).

2.3 Analogues as Probes of Substrate and Subtype Specificity

The combination of a growing library of analogues that potently bind to the EAATs, the ease with which the subtypes can be individually studied, and a crystallographic-based structural model of a homologous transporter have all had a dramatic impact on advancing our understanding of EAAT pharmacology. Two issues that have now progressed to the forefront of the
field concern delineating the structure–activity relations that govern subtype selectivity and substrate activity. The goal of developing subtype selective inhibitors is especially applicable to functional studies aimed at determining if the various EAATs, particularly EAATs 1–3 differentially contribute to the physiological roles generally ascribed to uptake, including signal termination, transmitter recycling, and excitotoxic protection. To date the greatest progress has been made in developing inhibitors that exhibit a marked selectivity for EAAT 2. These compounds include DHK and L-anti-endo-3,4-PDC among the pyrrolidine dicarboxylates, WAY-213394 and WAY-213613 among the aspartamides, PivA-TBOA and CNB-TBOA among benzoylaspartates, and the tricyclohexane analogue WAY-855 [32, 48, 56, 80]. While not as much progress has been made with respect to the other subtypes, new lead compounds are beginning to emerge. In the instance of the neuronal transporter EAAT 3, L-β-threo-benzyl-aspartate and NBI-59159 have been shown to preferentially block EAAT 3 with IC50/Ki values (0.8 and 0.1 µM, respectively) that are about 10 to 20 times lower than found with EAATs 1 or 2 [54, 81]. Both of these compounds function as non-substrate inhibitors. The triazole-based analogue AHTP is one of the first conformationally constrained analogues to exhibit a modest degree of selectivity for EAAT 1 (≈ 3-fold), compared to EAATs 2 or 3, although it is relatively weak when compared to the potency of the more recently developed blockers (IC50 ≈ 100 µM). Interestingly, substrate selectivity can also be used to distinguish EAAT 1 from EAAT 2. For example, L-serine-O-sulfate and 4-methylglutamate exhibit I_{max} values that are much more comparable to L-Glu at EAAT 1 (≈ 100 and 80%, respectively), than at EAAT 1, where L-serine-O-sulfate is only a moderate substrate (I_{max} ≈ 50%) and 4-methylglutamate is a non-substrate inhibitor. As a majority of the analogues discussed above have only been assessed for activity at EAATs 1–3, considerably less is known about the specificity of EAATs 4 and 5. Initial studies on these transporters have, however, identified a few distinguishing properties also linked to substrate specificity. For example the longer glutamate homologues L-α-aminoadipate and L-homocysteic acid that exhibit little or no interactions with EAATs 1–3, serve as substrates of EAAT 4 with I_{max} greater than L-Glu itself. In contrast, the substrate specificity of EAAT 5, is readily differentiated from the other subtypes by β-THA and L-trans-2,4-PDC, which are partial or full substrates of EAATs 1–4, but non-substrate inhibitors of EAAT 5.

It is quite intriguing that subtle variations among the “R” groups within families of compounds such as the benzoylaspartates, benzylaspartates, and aspartamides may be central to developing subtype selective inhibitors. SAR-based pharmacophore modeling with inhibitors of EAAT 1-3 and, more recently, crystallographic results, are consistent with the conclusion that the primary functional groups of the endogenous substrates (i.e., α-amino, α-carboxyl and distal carboxyl groups), are likely occupying very similar positions within the transporter binding sites. In turn, this would suggest that
the selectivity observed with the compounds described above may be due to the presence of lipophilic groups and the likelihood that the protein domains with which these groups interact vary among the subtypes. In particular, the structural data generated when the transporter (GLTțH) was crystallized with TBOA bound, suggest that the HP 2 loop thought to be involved in substrate gating may represent one of these lipophilic domains. However, the fact that a number of inhibitors, such as TBOA itself, act at all three EAATs and exhibit little or no selectivity, while the closely related PMB-TBOA and L-β-threo-benzyl-aspartate exhibit preferences for EAAT 2 and EAAT 3, respectively, indicates that it is not merely the presence or absence of a lipophilic R group that dictates specificity but probably subtle differences in its linkage, orientation and/or chemical composition.

The presence of the lipophilic R groups on these inhibitors also appears to be an important determinant in whether or not compounds act as alternative substrates for the transporters. Computational-based pharmacophore modeling with identified substrates demonstrated that these compounds not only share a very similar placement of amino and carboxylate groups, but also the space occupied by their respective carbon backbones [54]. Comparisons with other competitive inhibitors that lacked substrate activity allowed regions to be identified where steric bulk is tolerated within the binding site, but falls outside of the volume constrained by the substrates. In particular, there was considerable overlap in the regions occupied by the lipophilic R groups linked to the distal part of aspartate, such as present in the benzoylaspartates, benzylaspartates, and aspartamides. The increased potency of these inhibitors led to the hypothesis that the interaction of these lipophilic R groups with specific protein domains enhanced binding, but also led to a loss of substrate activity. Significantly, crystallographic studies with TBOA added a structural explanation to this concept, as the R-group of TBOA was shown to interact with the HP 2 loop of GLTțH and produce a configuration that was different from that observed in the “closed” orientation normally seen with the binding of aspartate [39]. Thus, the binding of TBOA (and its closely related analogues) may be enhanced because of its ability to interact with the HP 2 loop, but because this prevents the HP 2 loop from closing, the gating mechanism fails to operate and the inhibitor cannot be transported. It will be interesting to see if other non-substrate inhibitors, with smaller R-groups, (e.g., methyl-PDCs), can be utilized to define the limits of HP 2 loop movement during binding and translocation.

2.4 Analogues as Probes of Physiological and Pathological Roles

The EAATs are juxtaposed within the pathways of glutamate-mediated neurotransmission in such a manner as to potentially influence a number of variables, including: the concentration and time course with which vesic-
ularly released glutamate interacts with synaptic EAA receptors, spillover of transmitter out of the synaptic cleft and access to extrasynaptic receptors, and the general maintenance of extracellular levels of glutamate below those which could trigger excitotoxic pathology. Considerable effort has been focused on trying to determine how these various roles are distributed and/or differ among transporter subtypes, cell populations, and specific circuits. Toward this goal the inhibitors and substrates discussed above have often been used in attempts to assess whether or not the EAATs significantly contribute to a particular activity. For example, the questions as to the respective contributions of uptake and diffusion to the termination of the excitatory signal has been investigated by examining if excitatory postsynaptic current (EPSC) properties are effected by the presence of EAAT inhibitors. Such studies have revealed a heterogeneity among synaptic connections, as transport blockers produced a slowing of the EPSC in some circuits (e.g., inputs to cerebellar Purkinje neurons), but not in others (between CA 3 and CA 1 hippocampal neurons) [82–85]. Comparisons among a number of preparations suggest that transport will have more of an influence on excitatory signaling where there is an increased likelihood that L-Glu can accumulate in the synaptic cleft. These relatively higher levels of L-Glu could be a consequence of increased release due to the presence of multivesicular release, increased density of release sites, or increased incidence of high frequency stimulation. Alternatively, any spatial characteristic that would make the cleft less accessible to the bulk CSF, such as glial ensheathment, would tend to lessen diffusion and favor accumulation. Examples of connections in which EAAT-mediated uptake has been reported to contribute to signal termination include synapses of the cerebellar climbing fibers, mossy fibers, retinal bipolar ganglion, and auditory brain stem [86–91].

The localization of EAATs around synapses also places these transporters in a position to regulate the amount of L-Glu that can either escape from or enter a particular synaptic cleft. In this manner, uptake can potentially serve as a control point in determining the extent to which L-Glu released from one synapse can activate EAA receptors present presynaptically (i.e., spillover) or even in adjacent synapses (i.e., cross-talk). Once again, EAAT inhibitors have featured prominently in these studies and have been used to demonstrate that attenuating transporter activity can enhance the ability of L-Glu to reach and activate mGluRs at a distance. Examples include postsynaptic group I mGluR in the hippocampus and cerebellum, the group II mGluRs that mediate presynaptic inhibition on hippocampal neurons, and group III mGluRs on GABA terminals [92–94]. While it is more difficult to establish whether synaptic ionotropic receptors are being specifically activated by extrasynaptic L-Glu, studies suggest that this indeed is occurring and that the EAATs are influencing this signaling process in hippocampal CA1 cells, cerebellar stellate cells, and olfactory bulb mitral cells [95–98].
As will be discussed later in this review, the efflux of L-Glu through the System \( x_c^- \) (\( Sx_c^- \)) transporter rather than synaptic release may, in some brain regions, represent another physiologically relevant source of extracellular L-Glu. The normal operation of \( Sx_c^- \) stoichiometrically couples the import of L-cystine with the export of L-Glu [99]. In addition to providing a needed amino acid precursor, evidence suggests that under some circumstances the resulting extracellular L-Glu provides for the tonic activation of presynaptic mGluRs that, in turn, regulate L-Glu release [100–102]. Even though the exchange of L-cystine and L-Glu are mediated by \( Sx_c^- \), the EAATs play an integral role in both of these pathways. In the first, the EAATs maintain the L-Glu gradient necessary to drive the import of L-cystine, and in the second, it is the balance between the densities, location, and activities of both \( Sx_c^- \) and the EAATs that should ultimately determine if L-Glu accumulates to a sufficient level to activate the mGluRs.

As extracellular levels of L-Glu increase, the functional significance of the EAATs shifts from signal termination to excitotoxic protection. Both pharmacological and molecular manipulations of the EAATs indicate that there is an inverse relationship between transport activity and excitotoxic vulnerability. For example, co-administration of glutamate with transport inhibitors that exhibit little or no toxicity by themselves, such as L-2,4-PDC or \( \beta \)-THA, have been found to exacerbate the extent of the glutamate-mediated neuronal damage both in vitro and in vivo [103–106]. Subsequent studies also demonstrated that the pathological consequences of compromised EAAT activity was not necessarily dependent upon the inhibitors being co-administered with exogenous L-Glu. Thus, neuronal loss was also observed in long-term organotypic spinal cord slice cultures that were chronically exposed to EAAT inhibitors [107]. This study was of particular interest because of the reported loss of EAAT 2 in amyotrophic lateral sclerosis (ALS) (for review see [25]) and the observation that the motor neurons within these slices were found to be the most vulnerable to injury. Stereotaxic injections of TBOA directly into the hippocampus or striatum also produced increased extracellular levels of L-Glu, electroencephalographic activity indicative of seizures and neuronal damage [108]. In contrast, comparable administrations of L-2,4-PDC did not produce significant neuronal loss, suggesting that the substrate properties of an inhibitor (i.e., whether or not it can be cleared from the extracellular space, see above) can have a marked influence on its actions in vivo. The ability of an inhibitor to serve as a substrate also raises the possibility that the analogues may participate in the process of heteroexchange with intracellular L-Glu. This possibility was highlighted in experiments in which pathological conditions (e.g., typically energy depletion) lead to the disruption of the ion gradients used to maintain the L-Glu gradient and, consequently, promote its efflux through the reversed action of the transporter [48, 109–112]. Under such conditions, non-substrate inhibitors, such as TBOA and L-trans-2,3-PDC attenuate this reversed action, essentially trapping the transporter...
binding site in its external-facing configuration. Alternative substrates, on the other hand, can be translocated and allow a continued efflux of L-Glu as the transporter cycles between the external and internal compartments.

The pharmacological experiments described above are also consistent with studies in which the expression of the EAATs has been molecularly manipulated. Thus, neuronal pathology indicative of excitotoxicity was also observed following the in vitro or in vivo administration of antisense oligonucleotides for EAATs 1–3 [113, 114]. Mice that are homozygous deficient for Glt 1 (EAAT 2) show an increased vulnerability to cortical injury, hippocampal neurodegeneration, and die prematurely from severe spontaneous seizures [115]. GLAST (EAAT 1) knockout mice do not exhibit any overt neurodegeneration, but display motor problems and increased neuropathology following cerebellar injury [116]. Mice that are homozygous deficient for Glt 1 (EAAT 2) show an increased vulnerability to cortical injury, hippocampal neurodegeneration, and die prematurely from severe spontaneous seizures [115]. GLAST (EAAT 1) knockout mice do not exhibit any overt neurodegeneration, but display motor problems and increased neuropathology following cerebellar injury [116]. Similarly, neurodegenerative pathology was not present in mice homozygous deficient for EAAC 1 (EAAT 3), although there is a significant decrease in locomotor activity [117]. More recent studies on these mice report an increased neurodegeneration with age, decreased neuronal levels of glutathione and an increased vulnerability to oxidative stress, consistent with a role of EAAT 3 in cysteine transport and glutathione production [118]. Although brain development appeared normal in each of these transgenic mice, a GLAST/GLT 1 (EAAT 1/EAAT 2) double knock out revealed multiple deficits in cortical and hippocampal organization, indicating that there may be compensation by other subtypes in the individual mouse models [119].

Taken together, these pharmacological and molecular studies highlight the neuroprotective role of the EAATs and, consequently, have prompted a therapeutic interest in agents that enhance glutamate uptake, rather than inhibit it. Unfortunately, traditional structure activity studies and rational analogue design strategies lend themselves more to the development of inhibitors and substrates, rather than activating agents acting at a distinct site. That being said, a few potential strategies have emerged to enhance transporter activity. With respect to potential allosteric modulators, arachidonic acid has been reported to inhibit the activity of EAAT 1, but increase EAAT 2 mediated uptake by increasing its affinity for L-Glu [120]. More recently, attention has focused on the identification of molecules capable of targeting and activating specific gene promoters regulating EAAT expression. In the case of GLT 1 (the rat homologue of EAAT 2) a blind screen of 1,040 FDA approved drugs and nutritionals conducted as part of the NINDS Drug Screening Consortium identified a series of 10 to 15 β-lactam and cephalosporin antibiotics as potent stimulators of GLT1/EAAT 2 protein expression and activity. In particular, the addition of ceftriaxone to organotypic rat spinal cord slice cultures for 5–7 days resulted in a 3-fold increase in both cell surface GLT1 expression, as determined by immunoblotting and transport activity quantified by uptake of radiolabeled L-Glu [121]. Additionally, ceftriaxone was able to prevent glutamate induced large ventral mo-
tor neuron degeneration resulting from the chronic blockade of glutamate transport by \textit{threo}-β-hydroxyaspartate (THA) in organotypic spinal cord cultures. These findings point to possible therapeutic potential in neuropathologies where glutamate transport may be compromised elevating the risk of excitotoxicity.

3 System $\chi^-_c$: Cystine/Glutamate Exchanger

Much of the interest in the EAAT system has revolved around the process of uptake, that is the ability of these transporters to regulate extracellular L-Glu level by sequestering it intracellularly in glia or neurons. System $\chi^-_c$ ($\chi^-_c$), on the other hand, has attracted attention because its accepted mode of operation provides a route for the export of glutamate from cells into the extracellular environment of the CNS and, consequently, access to EAA receptors. As $\chi^-_c$ is an obligate exchanger, this efflux of L-Glu is linked with import of L-cystine (L-Cys$_2$), a sulfur-containing amino acid critical to a number of metabolic pathways, most notably the synthesis of glutathione (GSH) [122]. In this respect, both sides of this exchange reaction have significant implications within the CNS: the uptake of L-Cys$_2$ as a precursor in the maintenance of GSH levels for oxidative protection, and the efflux of L-Glu as a novel source of the neurotransmitter for excitatory signaling or excitotoxicity. The significance of these actions is reflected in the range of CNS processes to which $\chi^-_c$ has recently been linked, including: oxidative protection [123], the operation of the blood–brain barrier [124], drug addiction [102], and synaptic organization [125].

3.1 Properties

Like most transporters, $\chi^-_c$ was first identified (and named) on an activity dependent basis, where its specificity, ionic dependence, and exchange properties differentiated it from other transporters. Recent molecular studies reveal that $\chi^-_c$ is part of the heteromeric amino acid transporter family (HATs; aka glycoprotein-associated amino acid exchangers) [126, 127]. These proteins are disulfide-linked heterodimers containing one of two type-II membrane $N$-glycoproteins termed “heavy chains” (e.g., 4F2hc or rBat) in combination with one of several different highly hydrophobic, non-glycosylated “light chains”. In the instance of $\chi^-_c$, the heavy and light chains are 4F2hc ($\approx$ 80 kDa; aka CD98 or FRP1) and xCT ($\approx$ 40 kDa), respectively. The amino acid transport activity of the complex resides with the xCT subunit, which is thought to adopt a 12 transmembrane domain topology with the N and C termini found intracellularly. Thiol modification studies on xCT suggest
that a re-entrant loop between TM 2 and 3, as well as regions of TM 8 may be involved in substrate binding and permeation [128,129]. Interestingly, the xCT subunit has also been identified as the fusion-entry receptor for Kaposi’s sarcoma-associated virus [130]. The 4F2hc subunit has a single transmembrane domain and is required for the trafficking of xCT to the plasma membrane [128,131,132].

$S_x^-$ has been identified in a wide variety of CNS cells (e.g., astrocytes [99,133,134], microglia [135], retinal Muller cells [136], immature cortical neurons [137], and glioma cell lines [99,138]), as well as fibroblasts, macrophages, hepatocytes and endothelial cells [124,127,139,140]. The transporter is an obligate exchanger that mediates a 1:1 exchange of extracellular and intracellular amino acid substrates. In contrast to the EAATs, its activity is Na-independent and Cl-dependent, although it is still unclear if Cl– ions are co-transported. As mentioned above, its primary mode of operation couples the import of L-Cys$_2$ with the export of L-Glu, thereby allowing the L-Glu gradient generated by (EAATs) to serve as the driving force for L-Cys$_2$ accumulation. Activity can be assessed with either radiolabeled L-Cys$_2$ or L-Glu, with each acting as a competitive inhibitor of the other [99,138]. On the basis of the influence of pH, it was concluded that both L-Glu and L-Cys$_2$ are transported in an anionic form which, in turn, is also consistent with uptake being electroneutral [141].

3.2 Pharmacology

Initial pharmacological characterizations of $S_x^-$ defined the substrate activities of L-Cys$_2$ and L-Glu ($K_m \approx 50 \mu M$) (Fig. 8a,b), established it as an obligate exchanger and identified several defining features of its specificity, including that: (i) L-aspartate is neither a substrate nor inhibitor, (ii) homocysteate is an effective inhibitor, (i.e., the binding site will tolerate a SO$_3^-$ moiety in place of the distal COO$^-$ functional group) and (iii) $\alpha$-aminoadipate and $\alpha$-aminopimelate are inhibitors (i.e., the binding site will accept longer homologues of L-Glu) [142]. Ironically, some of the earliest insights into the presence and pharmacology of $S_x^-$ specifically within the CNS, emerged not from uptake studies, but from autoradiographic and membrane binding experiments. Thus, at a time when considerable effort was being devoted to the characterization of the EAA receptors, a chloride-dependent glutamate binding site identified in CNS preparations was suspected of being a novel receptor [143]. Subsequent studies, however, provided evidence that the observed binding was: likely associated with a transporter, could be blocked by L-Cys$_2$, exhibited a distinct distribution, and was enriched on glial membranes [144–148]. As the properties and roles of $S_x^-$ in other cell systems became more widely recognized, it became clear that much of this CNS binding was undoubtedly attributable to the $S_x^-$ transporter.
Exploiting the finding that CNS-derived tumor cell lines exhibit high levels of $\text{Sx}_-^-$ activity [99, 149–152] has allowed more thorough investigations of the structure–activity relationships (SARs) governing binding and translocation. Consistent with the inhibitory activity of L-homocysteate, the substrate-binding site accommodates the $\text{SO}_3^-$ moieties of $\text{S}$-sulfo-L-cysteine and L-serine-$\text{O}$-sulfate ($K_i \approx 25 \, \text{µM}$) (Fig. 8c), the $\text{SO}_2^-$ group of L-homocysteine-sulfinate, but not the $\text{PO}_4^{2-}$ group of L-serine-$\text{O}$-phosphate. Additionally, it appears as if an $N$-nitroso group can also serve as a $\gamma$-carboxylate mimic in the substrate binding site of $\text{Sx}_-^-$, as the antibiotic and antitumor drug L-alanosine (Fig. 8d) was identified as a competitive inhibitor in studies examining the transporter as a possible therapeutic target in the treatment of astrocytomas [153]. Assays demonstrating that the lathyris toxin $\beta$-$N$-oxalyl-$\text{L}$-$\alpha,\beta$-diaminopropionic acid ($\beta$-ODAP) (Fig. 8e) is also a competitive inhibitor about equipotent with L-Cys$_2$ provides further evidence that the distal portion of L-Glu can be derivatized and still retain activity at the transporter [151]. Additionally, both L-alanosine and $\beta$-ODAP can serve as alterna-

![Chemical Structures](image-url)
tive substrates for the transporter (see below) and exchange with intracellular L-Glu when incubated with cells expressing $S_x^-$. The ability to bind longer homologues of L-Glu, such as $\alpha$-aminoadipate (Fig. 8 f), is substantiated by the actions of S-carboxymethyl- and S-carboxyethyl-L-cysteine. However, there is a limit where increasing chain length to that of L-homocystine or L-djenkolate results in a marked loss of inhibitory activity [152].

The EAA ionotropic and metabotropic receptor agonists NMDA, KA, AMPA, L-AP4 and trans-ACPD were also all found to be essentially inactive as inhibitors, as were the Na$^+$-dependent EAAT inhibitors dihydrokainate and L-trans-2,4-PDC. While a lack of activity by these class-defining ligands clearly differentiate $S_x^-$ from the other glutamate system proteins, a number of conformationally constrained EAA analogues well-recognized for their interactions with EAA receptors have been identified as $S_x^-$ inhibitors. Probably the most prominent among these is quisqualate (QA) ($K_i \approx 5 \mu M$) (Fig. 8g) [99, 149, 152], which also acts as an agonist at AMPA and mGluR receptors [1]. The action of QA at $S_x^-$, as well as the significance of this transporter in providing L-Cys$_2$ for glutathione synthesis, was illustrated by the work of Coyle, Murphy and colleagues who demonstrated that certain neuronal cell lines and immature cortical neurons were vulnerable to QA-mediated toxicity because of compromised glutathione synthesis and oxidative stress, rather than a typical EAA receptor-mediated excitotoxic mechanism [137, 149]. Other EAA analogues that are now realized to exhibit some cross-reactivity with $S_x^-$, include: (i) ibotenate ($K_i \approx 30 \mu M$) (Fig. 8h) [152], an NMDA receptor [154] and non-selective metabotropic agonist [155], (ii) (RS)-4-Br-homoibotenate ($K_i \approx 20 \mu M$) [152], an AMPA receptor agonist [156] (iii) (RS)-5-Br-willardiine [152], a kainate receptor agonist [157] and (iv) 4-$S$-carboxy-phenylglycine (4-$S$-CPG; $K_i \approx 5 \mu M$) (Fig. 8i) [99, 149], a group I metabotropic receptor antagonist [158]. 4-$S$-CPG is of particular interest because it is one of the more potent inhibitors of $S_x^-$ yet identified and because it has been shown to control the growth of glial tumors that express high levels of the transporter [159]. More detailed studies with other phenylglycines revealed that 4-$R$-CPG was 1,000-fold less potent than the $S$-enantiomer and that S-4-carboxy-3-hydroxyphenylglycine and S-3-carboxy-4-hydroxyphenylglycine were also effective inhibitors as judged by the ability to block $S_x^-$-mediated release of L-Glu from tumor cells [160]. The lack of a carboxy group on the phenyl ring or the addition of a methyl group to the $\alpha$-carbon also resulted in a marked loss of inhibitory activity.

Even though the cross-reactivities of these compounds with other EAA proteins render them somewhat less valuable as physiological probes (as well as raise questions as to agonist-induced responses attributed to specific receptors) the analogues listed above begin to shed light on the SAR’s that govern the binding of L-Glu and L-Cys$_2$ to $S_x^-$. Thus, akin to what was observed with the EAATs, the requirement of an amino and carboxylate group in a classic L-$\alpha$-amino acid configuration appears almost absolute. Greater flexibility,
however, appears to be tolerated with respect to the position of distal carboxylate, which can be replaced with a variety of mimics. As many of the inhibitors entirely lack a distal carboxylate equivalent to the second amino group of L-Cys2, it may be that this functional group plays a less important role. The distances separating the carboxylate groups of L-Glu and L-Cys2, as well as in the configuration of charged groups in the conformationally restricted inhibitors (e.g., QA, 4-S-CPG, 3-S-4HPG) suggest that the binding site may tolerate some degree of variation with respect to placement of the distal acidic group. Indeed, given the strict requirement at the $\alpha$-position, it is tempting to speculate that the same domains on the transporter are interacting with the proximal functional groups on all the inhibitors, while there may be more than one domain participating in the binding of the distal charges. Given the shorter length of L-Glu (and a number of the other inhibitors) relative to L-Cys2, the binding domains for the two substrates may not be identical, but subsets of one another that exhibit the greatest overlap in the regions accommodating the $\alpha$ carboxylate and amino groups.

### 3.3 Substrate Specificity

As discussed with the EAATs, competition studies address the process of binding (evidenced by inhibition of uptake), but shed little insight as to whether an inhibitor is also a transportable substrate. Unlike the EAATs, however, uptake by Sx−c is not electrogenic, which limits the utility of electrophysiological recordings to quantify substrate translocation. Advantageously, the fluorometric assay originally developed to examine the vesicular release of L-Glu from synaptosomes can be readily modified to follow Sx−c activity [151, 152, 161]. Thus, the exchange of extracellular L-Cys2 for intracellular L-Glu through Sx−c can be quantified, in real time, by measuring the conversion of NADP+ to NADPH as the extracellular L-Glu is rapidly metabolized by glutamate dehydrogenase (GDH) included in the assay mixture. Using this approach the competitive inhibitors discussed above can be further differentiated on the basis of substrate activity. Once again, it appears that subtle structural changes in ligands can substantially influence not only binding, but also substrate activity. For example, ibotenate and serine-\(O\)-sulfate exhibit comparable $K_i$ values ($\approx 30 \mu M$) for inhibiting the uptake of $^3$H-L-Glu by Sx−c, yet ibotenate is transported as well as the endogenous substrate L-Cys2, while the substrate activity of serine-\(O\)-sulfate is only half as much [152]. QA, which is one of the most potent inhibitors of Sx−c ($K_i \approx 5 \mu M$), exchanges with L-Glu at a rate of about 35% of that observed with L-Cys2. The demonstration that QA can directly exchange for intracellular L-Glu through Sx−c is also consistent with its proposed role in “QA-sensitization” observed in physiological brain slice preparation, where internalized QA can be subsequently released through Sx−c and activate EAA receptors [162]. In contrast to these partial sub-
strates, 4-S-CPG also blocks $\text{Sx}_c^-$ with a $K_i \approx 5 \mu\text{M}$, but essentially fails to exchange with intracellular L-Glu. As appears to be the case with EAAT ligands, the most potent inhibitors identified tended to exhibit lower levels of substrate activity. This might reflect the fact that, as ligands are optimized for inhibition (and therefore binding), the compounds may be interacting strongly with domains that are distinct from those that participate in the translocation mechanism.

3.4 Analogues as Probes of Physiological and Pathological Roles

$\text{Sx}_c^-$ is a particularly intriguing transporter from a functional perspective, because both the import of L-Cys$_2$ and the export of L-Glu are each associated with a distinct set of physiological roles within the CNS. Until only recently, most studies on $\text{Sx}_c^-$ have primarily focused on its significance as a rate-limiting step in the provision of intracellular cysteine needed to maintain appropriate levels of the antioxidant glutathione (GSH). Given the sensitivity of the CNS to oxidative pathology it is not surprising that specific pathways have evolved to ensure neurons and glia have adequate capacities to produce GSH. Although both cell types require intracellular CysH to synthesize GSH, the extracellular precursor and route of entry necessary to provide this CysH appear to differ. Thus, a Cys$_2$/CysH shuttle has been proposed in which the CysH needed to maintain neuronal GSH levels is ultimately dependent upon the $\text{Sx}_c^-$-mediated uptake of Cys$_2$ into astrocytes and its subsequent efflux from the cell as either CysH or GSH [122, 163–165]. The significance of $\text{Sx}_c^-$ in GSH synthesis is underscored by the fact that QA-mediated toxicity in some cells is a consequence of blocking this transporter and the resultant oxidative stress, rather than the result of EAA receptor-mediated excitotoxicity [99, 149].

Within this protective context, $\text{Sx}_c^-$ expression is a part of a number of different adaptive cellular responses (e.g., amino acid starvation, oxidative stress, toxic exposure) that are under transcriptional control regulated by genomic cis-elements. In the most thoroughly studied cases, exposure to electrophiles and/or increased oxidative stress activates transcription factors (e.g., Nrf2) that bind to electrophilic-responsive elements (EpRE)/antioxidant-responsive elements (ARE) and result in the up-regulation of proteins presumed critical to detoxification and/or antioxidant defense mechanisms, including: GSH transferase, $\gamma$-glutamylcysteine synthetase, NAD(P)H: quinone reductase, heme-oxygenase 1 and $\text{Sx}_c^-$ [166–169]. The transporter is also present (and inducible) at the blood–brain barrier, where it may serve as a point of entry not only for L-Cys$_2$, but also structurally related drugs and neurotoxins (e.g., $\beta$-L-ODAP, see above) [124, 151, 164, 170]. Surprisingly, the level of $\text{Sx}_c^-$-mediated uptake typically reported to be present in primary cultures of astrocytes is remarkably low. This incon-
sistency was largely resolved when it was found that Sx\textsuperscript{−} activity is markedly up-regulated when the neonatal astrocyte cultures are differentiated with dibutyryl-cAMP and adopt a morphology more closely resembling that observed in vivo [134].

The Cys\textsubscript{2}/Cys\textsubscript{H} shuttle model, and its dependence upon Sx\textsuperscript{−} is, however, not without controversy. Indeed, it has been reported that the EAATs may provide another route of entry of Cys\textsubscript{2} into either neurons or glia [133, 171, 172]. Interestingly the EAATs, particularly neuronal EAAT 3, have also been postulated to transport Cys\textsubscript{H} selectively into neurons [173, 174]. This role has received considerable support with the finding that EAAT 3/EAAC 1-null mice exhibit markedly lower levels of neuronal GSH, increased markers of oxidative damage, enhanced sensitivity to oxidative stress, and exhibit age-dependent neurodegeneration [118].

As an obligate exchanger, the import of L-Cys\textsubscript{2} is coupled to the export of an equivalent amount of L-Glu. While this allows cells to utilize high intracellular concentrations of L-Glu as a driving force for the uptake of needed L-Cys\textsubscript{2}, it also provides a route for the accumulation of L-Glu in the extracellular space that, if not adequately regulated by the EAATs, could potentially trigger an excitotoxic response. Thus, in the two disorders where the evidence is strongest for Sx\textsuperscript{−} acting as a source for excitotoxic levels of L-Glu, CNS infection and glial tumors, it is not surprising that both of the cell types involved express enriched levels of the transporter. In CNS infection and inflammation, the cellular source of L-Glu are microglia, whose oxidative-based defense mechanisms necessitate high levels of Sx\textsuperscript{−} activity to provide the Cys\textsubscript{2}/Cys\textsubscript{H} necessary to maintain an adequate glutathione supply. Ironically, it thus appears that the excitotoxic pathology associated with a number of infections may actually be a secondary result of the migration of high numbers of microglia into an area and a subsequent release of L-Glu as these cells import the L-Cys\textsubscript{2} integral to their protective roles [135, 175].

Similarly, astrocytoma cells express markedly higher levels of Sx\textsuperscript{−} [153, 160]. Numerous cellular and functional changes accompany the progression from low-grade astrocytomas to high-grade glioblastoma multiforme tumors. Of particular interest is the observation that the increase in Sx\textsuperscript{−} expression is also accompanied by a reduction in EAAT-mediated transport of glutamate [138]. Several studies utilizing human tumor cell lines and animal models now suggest these tumors employ the mechanism of excitotoxicity, in part by the release of excessive glutamate through Sx\textsuperscript{−}, to actively kill surrounding neurons in the peritumoral space to aid tumor expansion [138, 160, 176]. Epileptic seizures associated with brain tumors are also believed to result from this released glutamate. Further, the concurrent ability to acquire more L-Cys\textsubscript{2} and maintain high levels of GSH allows the tumor to survive the necrotic biochemical environment engulfing the tumor. As a transporter, Sx\textsuperscript{−} may also impact the chemosensitivity and chemoresistance of a broad range of tumors through the uptake and efflux of anticancer
agents [153, 159, 177]. The application of identified Sx\textsubscript{c} inhibitors, (S)-4-CPG or sulfasalazine (Fig. 8j) to a series of glioma cell lines produced a marked reduction in L-Cys\textsubscript{2} uptake and intracellular GSH concentration whilst also inhibiting tumor cell growth and inducing caspase-dependent apoptotic cell death. Sulfasalazine was also demonstrated to suppress tumor growth in vivo. In addition to inhibiting the uptake of L-Cys\textsubscript{2}, L-alanosine has been shown to induce cytotoxicity in lung and ovarian cancer cell lines as a consequence of its intracellular accumulation following uptake through Sx\textsubscript{c}. Consistent with such a mechanism, inhibition of Sx\textsubscript{c} with S-4-CPG decreased the cytotoxicity of L-alanosine. In contrast, the cytotoxic potential of geldanamycin was increased following inhibition of Sx\textsubscript{c}, as the subsequent reduction in GSH level attenuated the detoxification of this drug. This suggests a multidrug strategy based on substrate and/or inhibitor activity may be a relevant therapeutic approach in treating tumors that needs to be further investigated.

Significantly, evidence is beginning to emerge that this export of L-Glu through Sx\textsubscript{c} may be relevant to more than just pathological mechanisms and may actually represent a novel route of release through which L-Glu can activate extrasynaptic EAA receptors. Based primarily on microdialysis data, work by Kalivas, Baker and coworkers report that Sx\textsubscript{c} appears to be a primary source of extracellular L-Glu in select brain regions, such as the striatum and nucleus accumbens [100–102]. Indeed in vivo microdialysis has revealed Sx\textsubscript{c} is the primary source of non-vesicular extracellular glutamate outside the synaptic cleft and responsible for as much as 50–70% of the basal extracellular level in the nucleus accumbens (NAc). Consistent with this hypothesis, inhibition of Sx\textsubscript{c} with 4-S-CPG reduced extracellular L-Glu level, as well as blocked the accumulation of L-Glu produced by inhibiting EAAT activity. This efflux of L-Glu is thought to regulate synaptic release (of both L-Glu and dopamine) through the tonic activation of extrasynaptic Group II mGluRs, which have been implicated in various forms of neuroplasticity and neurophychiatric disorders. Of particular significance, these extracellular levels of L-Glu are reduced in the nucleus accumbens during cocaine addiction and withdrawal, an effect attributed to the decreased function of Sx\textsubscript{c} [102]. Consistent with this model, the infusion of Cys\textsubscript{2} restored L-Glu levels and, importantly, treatment of rats with the CysH/Cys\textsubscript{2} prodrug N-acetyl-CysH prevented reinstatement in cocaine-addicted rats. This led to the conclusion that the increased susceptibility to relapse that accompanies withdrawal from cocaine addition is linked to the decreased activity of Sx\textsubscript{c}, reductions in extracellular L-Glu levels, and consequent loss of mGluR-mediated regulation of excitatory transmission. Remarkably restoring Sx\textsubscript{c} activity, presumably with increased substrate levels, prevents “cocaine-primed drug seeking” [102, 178]. This suggests that in addition to receptor-targeted approaches, agents directed at Sx\textsubscript{c} may hold therapeutic value in the treatment of addiction.

This postulated role of Sx\textsubscript{c} (and Cys\textsubscript{2}) in regulating extracellular L-Glu level is, however, not without its controversies and complications. For ex-
ample, very similar microdialysis studies in prefrontal cortex demonstrated that inhibition of \( Sx^-c \) did not reduce extracellular L-Glu concentrations, yet did block the accumulation of L-Glu produced following the inhibition of EAAT activity [179]. All of these studies are also complicated by the fact that the \( Sx^-c \) inhibitor most often employed was 4-S-CPG, which is also an mGluR1/5 antagonist. Ironically, during the course of the studies another mGluR1/5 antagonist (LY367385) was also found to be an \( Sx^-c \) inhibitor [179]. In related studies by Atwell and coworkers, an L-Cys\(_2\)-mediated efflux of L-Glu through \( Sx^-c \) was shown to be significant enough to activate non-NMDA in cerebellar slices and NMDA receptors in hippocampal slices [180, 181]. However, it was concluded that given both CSF levels of L-Cys\(_2\) (typically in the 0.1–0.5 \( \mu \)M range) and its \( K_m \) values at \( Sx^-c \) (typically reported in 100 \( \mu \)M range), it was unlikely that this mechanism contributed to tonic glutamate levels (and EAA receptor signaling) under normal physiological conditions. This conclusion, however, must be tempered somewhat by direct measurements of \( ^{35}S \)-L-Cys\(_2\) uptake in slices of nucleus accumbens which yielded \( K_m \) values in the 2–4 \( \mu \)M range [102]. Further, an \( Sx^-c \)-mediated efflux of L-Glu was also shown capable of decreasing the synaptic release of L-Glu (decreased mEPSC and spontaneous EPSC frequency) as a consequence of presynaptic mGluR2/3 activation by levels of L-Cys\(_2\) in the 0.1–0.3 \( \mu \)M range [178]. Lastly, recent studies in which homologues of \( Sx^-c \) (e.g., genderblind) have been genetically eliminated in \textit{Drosophila} support the participation of \( Sx^-c \) in the regulation of extracellular L-Glu levels and further suggest a role of this extracellular L-Glu in iGluR desensitization and clustering [125].

Taken together, these findings suggest that \( Sx^-c \) may indeed function to regulate extracellular L-Glu levels and set a tone at extrasynaptic mGluRs, although this contribution may be a function of the synaptic circuit being examined, the activity of \( Sx^-c \) and EAATs, as well as extracellular Cys\(_2\) levels. A particularly intriguing aspect of these variables concerns the balance set between these two transporters, with respect to kinetic properties, expression levels and localizations within the microenvironment of extrasynaptic receptors. Changes in any one of these properties, whether the result of development, plasticity, or pathology could impact excitatory signaling and/or excitotoxic vulnerability. Assessing the coordinate activity and functions of the EAATs and \( Sx^-c \) will be dependent upon the continued development of potent, selective inhibitors and substrates in combination with more thorough understanding of the structure–activity relationships that govern binding and uptake.

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