1999

Structure-activity relationships of the high-affinity glutamate transporters in synaptosomes

Hans-Peter Koch
The University of Montana

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

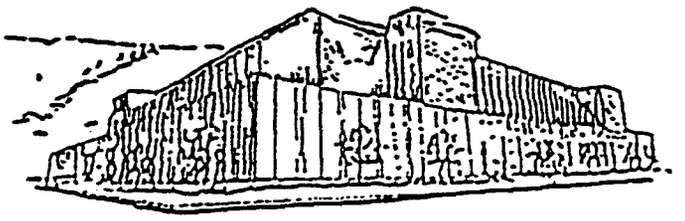
In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI®

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600



Maureen and Mike
MANSFIELD LIBRARY

The University of **MONTANA**

STRUCTURE-ACTIVITY RELATIONSHIPS OF THE HIGH-AFFINITY GLUTAMATE TRANSPORTERS IN SYNAPTOSOMES

by

Hans Peter Koch

B.S. University of California, Irvine 1993

Presented in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

Department of Pharmaceutical Sciences

The University of Montana

1999

Approved by:

Chairperson

Dean, Graduate School

5-28-99

Date

Koch, Hans P., Ph.D. May, 1999 Pharmaceutical Sciences

Structure-activity relationships of the high-affinity glutamate transporters in synaptosomes.

Research advisor: Richard J. Bridges, Ph.D.

Within the mammalian central nervous system, the efficient removal of the neurotransmitter L-glutamate from the extracellular space by excitatory amino acid transporters (EAATs) has been postulated to contribute to signal termination, the recycling of transmitter, and the maintenance of L-glutamate at concentrations below those which are excitotoxic. In the present study, several novel series of L-glutamate analogues, including the 2,3-pyrrolidine dicarboxylates and 3,4-methanopyrrolidine dicarboxylates, were investigated for their activity as inhibitors of the high-affinity glutamate transport system present in rat forebrain synaptosomes. Further, these novel analogues, as well as a library of previously identified uptake inhibitors, were used to address two key issues: the differentiation of substrates from nontransportable inhibitors and the comparison of the pharmacological profile of synaptosomal uptake with those of the individual EAAT clones. It was demonstrated that the process of transporter-mediated heteroexchange could be exploited in synaptosomes in order to rapidly distinguish transportable from nontransportable inhibitors. Using this approach, we identified 2,4-methanopyrrolidine-2,4-dicarboxylate, *cis*-1-aminocyclobutane-1,3-dicarboxylate, and L-*trans*-2,4-pyrrolidine dicarboxylate as substrates for the synaptosomal glutamate uptake system. In contrast, L-*anti-endo*-3,4-methanopyrrolidine dicarboxylate, L-*trans*-2,3-pyrrolidine dicarboxylate and dihydrokainate proved to be competitive inhibitors of glutamate transport that exhibited little or no activity as substrates. When these same compounds were electrophysiologically characterized for substrate activity in *Xenopus* oocytes expressing the human transporter clones EAAT1, EAAT2 or EAAT3, it was found that the pharmacological profile of the synaptosomal system exhibited the greatest similarity with the EAAT2 subtype, a transporter believed to be expressed primarily on glial cells. In the final portion of the study, we examined the efflux of excitatory amino acid in response to a metabolic insult (5 mM KCN and 1 mM iodoacetate). Exposure of synaptosomes containing [^{3}H]-D-aspartate to the metabolic inhibitors increased the efflux of the radiolabeled substrate to over 200% of control values. The inclusion of L-*trans*-2,3-pyrrolidine dicarboxylate and dihydrokainate, both identified as nontransportable uptake inhibitors, significantly attenuated the metabolic inhibitor-induced efflux. These findings add further strength to the model of CNS injury-induced efflux of L-glutamate through its high-affinity transporters and identify a novel strategy to attenuate this process.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

As any reasonable athlete in organized sports these days knows, victory is achieved as a result of teamwork and not by one player's efforts alone. The same, of course, can be said about efforts in the sciences. In this regard, the greatest acknowledgment for this project belongs to the "M.V.P." of the team, Dr. Richard Bridges, for he is an individual who often plays the dual roles of both teammate and leader, bringing strength to the organization through guidance while at the same time setting up the other athletes for the big plays. Fans will also recognize the greatness of the coaching staff of a winning organization, and I would thus like to express my gratitude to Dr. Vernon Grund, Dr. Keith Parker, Dr. Charles Thompson, and Dr. Richard Chamberlin for their invaluable advice and insightful discussions. Special thanks to Dr. Sean Esslinger for his assistance with the molecular modeling and in preparing the manuscripts, and to Dr. Michael Kavanaugh for sharing his work on the electrophysiology and molecular biology of the transporter clones. I would like to express my appreciation for the "league", the Department of Pharmaceutical Sciences, for giving me the opportunity and the support to study in this wonderful program. Thanks to my teammates Colleen Baker, Jennifer Hensley and Taren Grass for their technical contributions to the project. I would also like to acknowledge my personal heroes Skip Horner, Dr. Michael Anderson, Dr. Daniel Fox and Larry Palting for

keeping me pointed in the right direction. Thanks to Sarah Potoczny for her love and support throughout this project. And finally, I would especially like to thank my mother Sylvia and father Bruno for giving me the world and being the best parents a guy could have.

Chapter 1: Background and Introduction

Nearly three decades passed between the original experiments demonstrating that L-glutamate could cause neuronal excitation (Curtis et al., 1959) and the eventual acceptance of this α-amino acid as the primary excitatory neurotransmitter in the mammalian central nervous system (CNS). The reluctance to acknowledge L-glutamate as a transmitter candidate was due, at least in part, to its omnipresence in virtually all biological processes as well as its diverse roles as a building block in the synthesis of peptides and proteins, and as a central molecule in intermediary metabolism. L-Glutamate is now recognized as one of the class of amino acid neurotransmitters that accounts for the majority of all transmission within the mammalian CNS. As the primary excitatory amino acid (EAA) transmitter, L-glutamate has been found to participate in standard fast synaptic transmission as well as the higher order signal processing required for development, plasticity, learning and memory (for review see: Cotman et al., 1995).

L-Glutamate mediates these functions through the activation of multiple EAA receptor classes (Figure 1.1), including the ionotropic (ion channel-coupled) and metabotropic (second messenger-coupled) subtypes. The ionotropic receptors were initially identified and distinguished pharmacologically by the selective agonists for which they were named, i.e., N-methyl-D-aspartate (NMDA), kainic acid (KA), and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA). These receptors typically gate currents carried by Na^+, K^+, and often times Ca^{2+} ions (for review, see: Dingledine et al., 1999). The metabotropic glutamate

receptors (mGluRs) were originally characterized and named after the agonists *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*trans*-ACPD) and L-2-amino-phosphonobutyric acid (L-AP4), and are coupled via guanine nucleotide binding proteins (G proteins) to either 3',5'-cyclic adenosine monophosphate (cAMP) or phosphoinositol (PI) second messenger systems (Conn and Pin, 1997).

In stark contrast to the normal processes mediated by this neurotransmitter, over-stimulation of the EAA receptors by excessive levels of L-glutamate can result in a type of neuronal damage known as excitotoxicity (Rothman and Olney, 1987; Choi, 1988; Choi, 1994; Rothman and Olney, 1995). The first component of this pathology, or the acute phase, is initiated by the influx of excessive amounts of Na^+ through activated EAA receptors. This is then followed by the passive influx of Cl^- and water, and thus consequent cell swelling. The second component is thought to be associated with more long term neurodegeneration and revolves around the loss of Ca^{2+} homeostasis. Excess intracellular Ca^{2+}, most likely a result of influx through activated NMDA receptors, as well as release from intracellular stores, has been shown to result in the activation proteolytic and lipolytic enzymes, increase in free radical production, apoptosis, and eventual cellular degeneration (for review, see: Choi, 1995). Excitotoxic neuropathology is thought to be a factor in a number of CNS pathologies., including acute CNS injuries such as stroke, ischemia, anoxia and trauma, as well as chronic neurodegenerative syndromes such as amyotrophic lateral sclerosis (ALS), Huntington's disease, and Alzheimer's disease (Choi, 1994; Rothman and Olney, 1995; Leigh and Meldrum, 1996; Arzberger et al., 1997; Olney et al., 1997).

The balance between the physiological and pathological actions of L-glutamate is thought, at least in part, to be kept in check by the rapid removal of this acidic amino acid from the synaptic cleft. While a number of systems have been identified that are capable of transporting L-glutamate into cells, the majority of uptake within the CNS appears to be mediated by the high-affinity, sodium-dependent excitatory amino acid transporters (EAATs) (for review see: Gegelashvili and Schousboe, 1997; Vandenberg, 1998). Transport of L-glutamate by the EAATs is electrogenic and is driven primarily by the trans-membrane sodium gradient generated by Na^+/K^+ ATPases (for review see: Rudnick, 1998). The efficient removal of L-glutamate from the extracellular space by these systems has been postulated to contribute to signal termination, the recycling of the transmitter, and the maintenance of L-glutamate at concentrations below those which are excitotoxic (for review see: Takahashi et al., 1997).

Several subtypes of sodium-dependent glutamate transporters have been isolated and cloned from mammalian tissue, including GLAST and GLT-1 from rat brain, and EAAC-1 from rabbit intestine (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992). The human homologues of these transporters, EAAT1, EAAT2 and EAAT3, respectively, have also been identified (Arriza et al., 1994). Studies of cellular expression indicate that EAAT1/GLAST and EAAT2/GLT-1 are present primarily on astrocytes, whereas the EAAT3/EAAC-1 subtype is preferentially found on neurons (for review see: Gegelashvili and Schousboe, 1998). More recent investigations have revealed two additional human subtypes: EAAT4, which is selectively found in cerebellar tissue, and EAAT5, a retinal transporter (Fairman et al., 1995;

Arriza et al., 1997). Cloning of the transporters provided the means with which to individually express the proteins in cell systems, such as *Xenopus* oocytes, and hence allowed for the delineation of more precise functional characteristics. Current modeling of EAAT stoichiometry suggests that L-glutamate is co-transported with 3 sodium ions and a proton in exchange for one potassium ion (Figure 1.2), and that uptake is also associated with a non-thermodynamically coupled chloride conductance (Zerangue and Kavanaugh, 1996; Levy et al., 1998).

Our understanding of the pharmacological specificity of these transporters, as well as their functional roles, has benefited greatly from the development of potent and selective inhibitors. Conformationally constrained analogues of L-glutamate, like those that proved invaluable in the delineation of the EAA receptors, have been especially useful in this regard (Chamberlin et al., 1998). The acyclic structure of L-glutamate allows for a good deal of folding and rotation of the molecule and thus the assumption of a wide range of conformations. As a consequence of their restricted flexibility, the constrained analogues are capable of mimicking only a very limited number of the conformations that are attainable by L-glutamate. Not only does this allow for the positions of the functional groups required for binding to the systems to be defined, it also decreases the number of binding sites the compound can occupy, thereby increasing selectivity. Often these compounds are designed with the goal of embedding L-glutamate or L-aspartate (or a portion thereof) into a ring system. This strategy led to the development of uptake inhibitors such as L-*trans*-2,4-pyrrolidine dicarboxylate (L-*trans*-2,4-PDC), (2S,3S,4R)-2-(carboxycyclopropyl)glycine (L-CCG-III), *cis*-1-aminocyclobutane-1,3-

dicarboxylate (*cis*-ACBD), and D,L-β-*threo*-hydroxyaspartate (D,L-β-THA) (Figure 1.3). Initially used to characterize the pharmacology of glutamate uptake in physiological preparations such as synaptosomes, tissue slices and cell cultures, these inhibitors have more recently been employed in expression systems and used to probe the specificity of the cloned transporters (Arriza et al., 1994; Yamashita et al., 1995; Esslinger et al., 1998).

Ironically, under some pathological conditions the same high-affinity, sodium-dependent uptake systems that normally maintain a normoxic synaptic environment may actually participate in the excitotoxic process by acting as sites of efflux of L-glutamate from intracellular compartments (Takahashi et al., 1997). Numerous studies have suggested that metabolic insults which compromise cellular energy levels can lead to the reversed action of the transporters and the movement of L-glutamate down its concentration gradient into the extracellular space (Kauppinen et al., 1988; Sanchez-Prieto and Gonzalez, 1988; Gemba et al., 1994; Szatkowski and Attwell, 1994; Longuemare and Swanson, 1995). This pathway, possibly in combination with alternative routes of L-glutamate efflux (Kimelberg et al., 1990), could contribute to the rise in extracellular glutamate levels observed in models of anoxia and ischemia (Benveniste et al., 1984; Roettiger and Lipton, 1996).

The work detailed in the present study was conducted essentially in three phases. First, several series of novel conformationally constrained glutamate analogues, including 2,3-pyrrolidine dicarboxylates, 3,4-methanopyrrolidine dicarboxylates and 2,4-methanopyrrolidine dicarboxylate, were tested for the ability to inhibit the high-affinity,

sodium-dependent transport system present in forebrain synaptosomes. Detailed studies were included to kinetically characterize the potent inhibitors. The second phase of the study tested the hypothesis that analogues could be classified either as substrate or nonsubstrate inhibitors of the high-affinity transporters. In other words, it was presumed that a distinction could be made between compounds that compete with L-glutamate for the binding site and are then transported (substrate inhibitors), and inhibitors that compete for binding but are not translocated (nontransportable inhibitors). This was accomplished by utilizing the process of transporter-mediated heteroexchange, in which an externally applied substrate, such as L-glutamate, stimulates the efflux of an internal substrate, e.g. D-aspartate, that had been previously sequestered into the synaptosomes. Thus, in lieu of radiolabeling the individual analogues, transporter-mediated heteroexchange could be efficiently exploited to evaluate a large number of compounds for substrate activity. The final phase of the study tested the hypothesis that high-affinity glutamate transporters could reverse direction during a metabolic insult and release amino acid into the extracellular space. This was accomplished by exposing synaptosomes containing radiolabeled D-aspartate to a simplified chemical model of ischemia/anoxia. The combination of potassium cyanide and iodoacetate was used because of its ability to effectively inhibit respiration, deplete ATP levels, and erode synaptosomal membrane potentials. Exposure of the synaptosomes to this metabolic insult increased the efflux of radiolabeled substrate to over 200% of control values. Inclusion of the nontransportable uptake inhibitors L-*trans*-2,3-pyrrolidine dicarboxylate and dihydrokainate significantly

attenuated the metabolic inhibitor-induced efflux. This finding not only provides new insight into the transport mechanism but also adds further strength to the model of CNS injury-induced efflux of L-glutamate through the high-affinity transporters.

Specific Aims

The specific aims of this project will be to:

1) Delineate the pharmacophore of the sodium-dependent, high-affinity glutamate transporter by using a library of novel, conformationally constrained glutamate analogues that are based on the pyrrolidine dicarboxylate (PDC) structure. This structure-activity data will then be used to develop a pharmacophore model for the synaptosomal glutamate transporter.

2) Determine if the inhibitors identified in Specific Aims 1 are substrates or nontransportable inhibitors of the synaptosomal glutamate carrier by utilizing the process of heteroexchange.

3) Kinetically characterize the efflux of amino acid neurotransmitters from synaptosomes observed during a metabolic injury and compare this process with that seen during heteroexchange.

4) Quantify the ability of substrates and nontransportable inhibitors of glutamate uptake to alter the metabolic injury-induced efflux of amino acid neurotransmitters from synaptosomes.

Figure 1.1: Excitatory Amino Acid Synapse

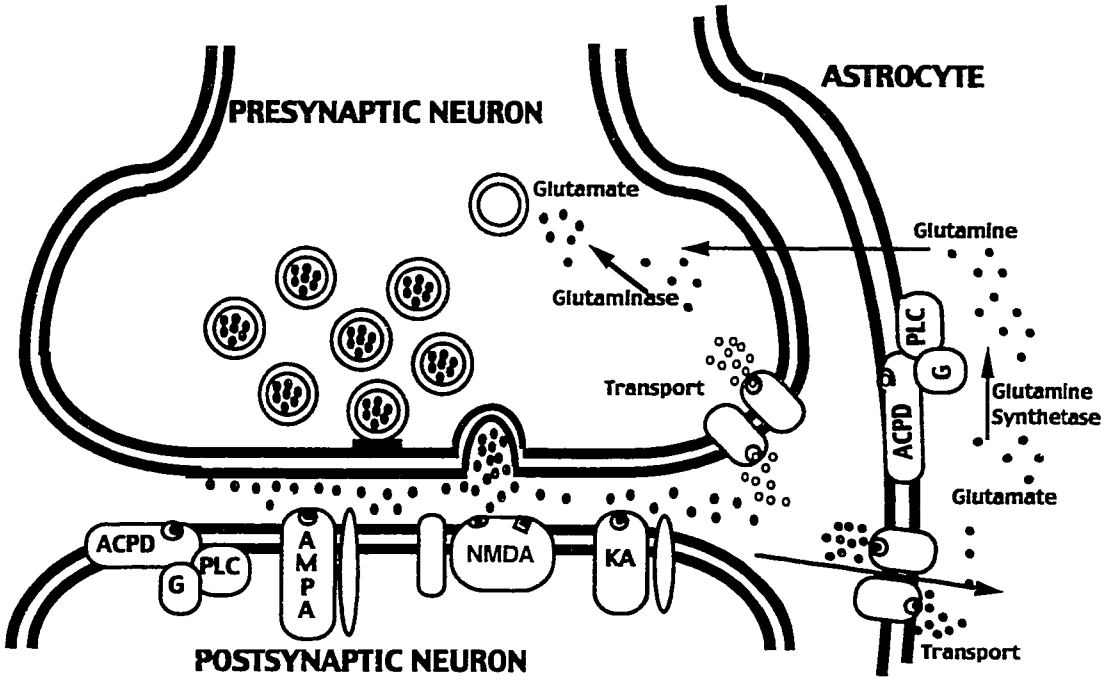
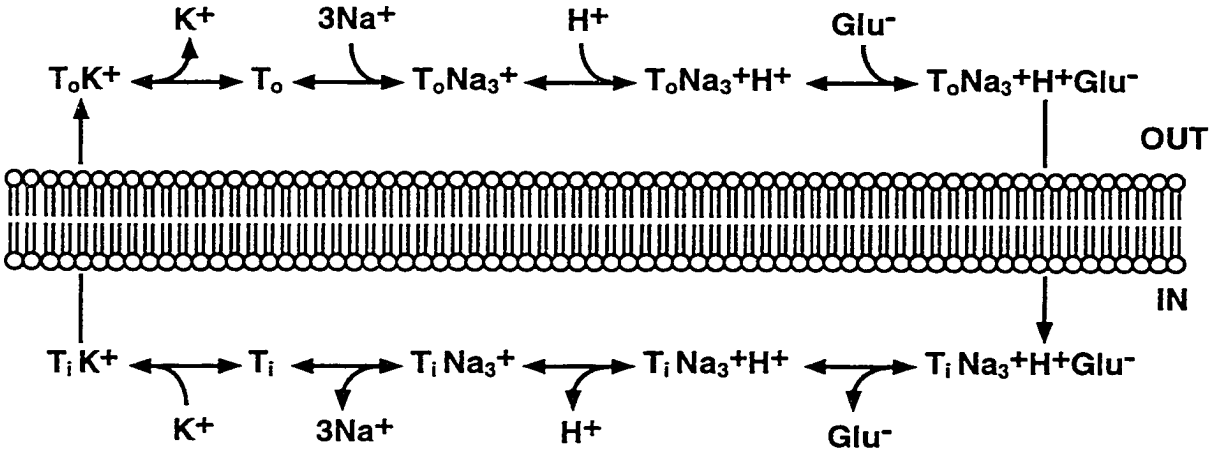
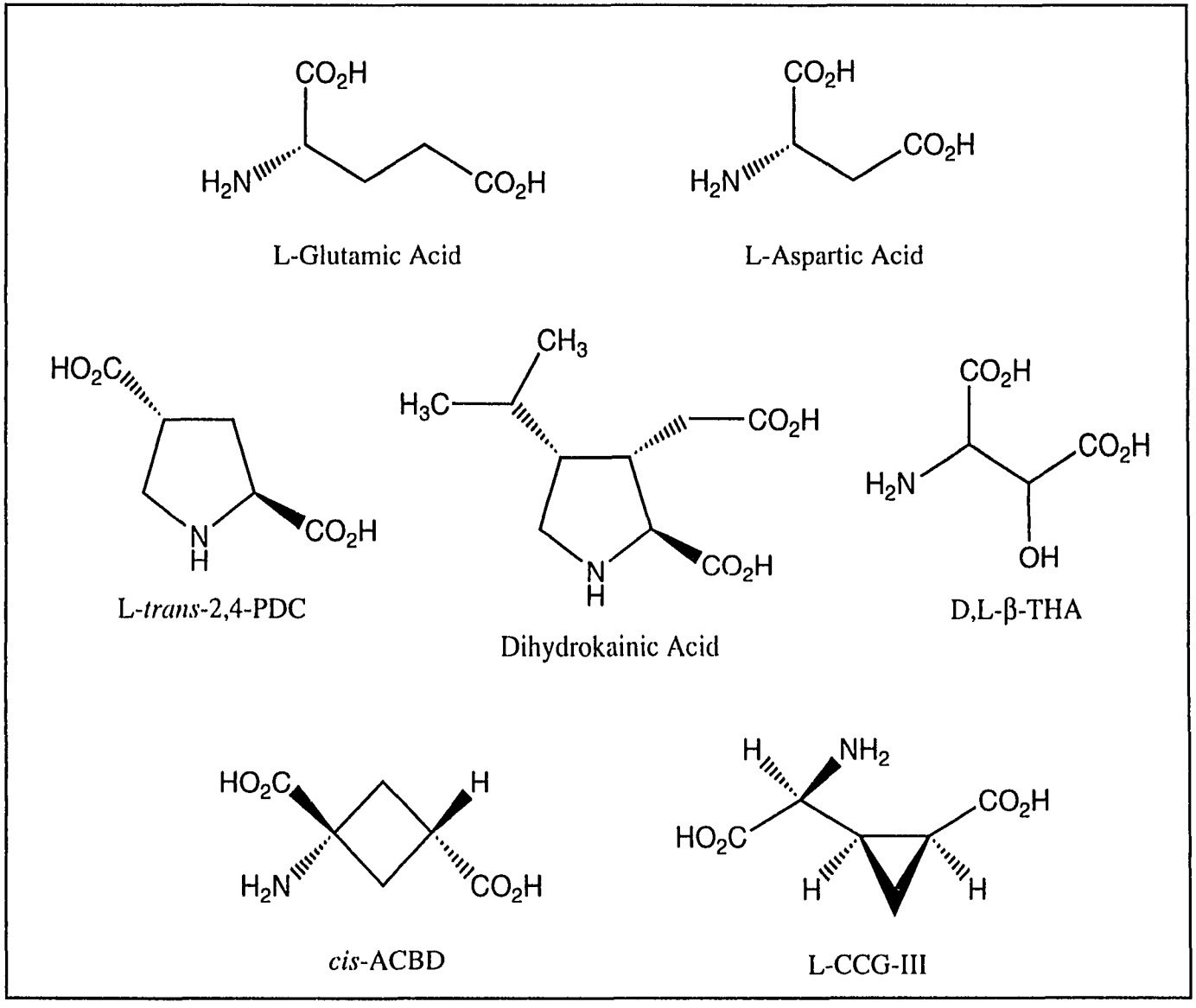


Figure 1.2: Proposed Stoichiometry for Na^+-Dependent Glutamate Transport



A current model for high-affinity, sodium dependent uptake of L-glutamate (Glu^-) suggests that 3 sodium ions must first bind to the transporter on the extracellular surface (T_O), followed by the sequential binding of one proton and one molecule of L-glutamate. The movement of the transporter to the intracellular surface (T_i) is then followed by the sequential unbinding of L-glutamate, the proton, and the three sodium ions. The transporter then returns to the extracellular surface with one potassium ion, which must then unbind in order for another cycle of transport to begin (Zerangue and Kavanaugh, 1996).

Figure 1.3: Conformationally Constrained L-Glutamate Analogues



Chapter 2: Methods and Materials

The development of conformationally constrained inhibitors of the glutamate transporters, as well as the testing of proposed pharmacophore models, is ultimately dependent upon assessing the ability of the analogues to bind to the substrate site on the transport proteins. This has typically been accomplished by quantifying the capacity of analogues to competitively inhibit the uptake of a radiolabeled substrate (e.g., [^{3}H]-L-glutamate or [^{3}H]-D-aspartate) into any one of a number of CNS preparations (e.g., brain slices (Balcar and Johnston, 1972), primary cultures of neurons or glia (Garlin et al., 1995), or synaptosomes (Bridges et al., 1994). As synaptosomes can be rapidly prepared and easily used to screen large numbers of analogues, this preparation has been widely employed in structure-activity studies (Bridges et al., 1991; Fletcher et al., 1991; Nakamura et al., 1993).

While the various preparations of CNS tissue may provide a more relevant pharmacological overview of transport as it occurs *in vivo*, the systems do suffer from potential complications associated with heterogeneity. As an alternative, the recent cloning and expression of distinct glutamate transporters has allowed each of the subtypes to be selectively characterized. Advantageously, expression of the individual transporters in oocytes permits uptake to be quantified both biochemically and electrophysiologically (Arriza et al., 1994). Measurement of uptake-induced currents provides an approach to assess the suitability of glutamate analogues as transporter substrates that is not dependent upon the availability of radiolabeled analogues. In contrast, it must be

remembered that competitive inhibition studies with radiolabeled substrates address only whether or not an analogue binds to the transporter, not if it is also translocated. This limitation can be in part overcome in more traditional neurochemical preparations by testing for the process of heteroexchange. Early studies of glutamate uptake demonstrated that the addition of an alternative substrate could stimulate the efflux of another substrate that had previously been transported ("loaded") into the synaptosomes (Erecinska and Troeger, 1986). Thus, if an analogue is able to undergo heteroexchange, it can be further classified as a substrate and differentiated from nontransportable inhibitors that bind to the substrate site on the carrier protein, but are not translocated. Caution must be exercised in these experiments to insure that the initial internal content of radiolabeled substrate is similar when comparisons of efflux rates are made and that the observed efflux occurs specifically via the transporter and not by a secondary mechanism (e.g., nonspecific leakage, receptor activated event). The specificity of the leakage can be confirmed with a previously identified nontransportable inhibitor (e.g., dihydrokainate, (Arriza et al., 1994)) and the potential contribution of EAA receptors can be excluded through the use of EAA agonists. The process of exchange has recently been used to demonstrate that the conformationally constrained analogues L-*trans*-2,4-PDC (Bridges et al., 1991) and *cis*-ACBD (Fletcher et al., 1991) are transportable inhibitors of high-affinity glutamate uptake in cerebellar granule cells and cortical astrocytes (Griffiths et al., 1994) and that L-*trans*-2,3-PDC is a nontransportable inhibitor of the high-affinity glutamate transporter in forebrain synaptosomes (Willis et al., 1996).

Synaptosomal Preparation:

Numerous procedures are available for the preparation of CNS synaptosomes (Robinson, 1998). The following protocol, which employs a discontinuous Ficoll gradient, is based upon the method of Booth and Clarke (1978).

Reagents:

Homogenization Buffer (HB): 10 mM Tris-acetate, 0.32 M sucrose, pH 7.4 (glacial acetic acid)

Ficoll: (Type 400 DL, Sigma) 7.5%, 12.0% , 0.32 M sucrose, 40 μM EDTA (potassium salt), pH 7.4 (KOH)

Assay Buffer (AB): 10 mM Tris-base, 128 mM NaCl, 10 mM D-glucose, 5 mM KCl, 1.5 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7.4 (glacial acetic acid)

Two male Sprague-Dawley rats were sacrificed by rapid decapitation. The brains were dissected free from the skull, the cerebellums removed, and the tissue placed into ≈20 ml of ice cold HB. The HB was decanted and the tissue resuspended in fresh, ice cold HB (10 ml/brain). While submerged in the HB, the forebrains were diced into small pieces with

scissors and then transferred to a chilled Wheaton glass/glass homogenizer (15 ml, 0.001"-0.003" clearance). The tissue was homogenized by hand (12 strokes) and then centrifuged (1100 *g*, 3 min, 4 °C, Sorvall SS-34 rotor). The supernatant was collected and recentrifuged (14,600 *g*, 10 min, 4 °C). The resulting pellet was resuspended in 10 ml 12% Ficoll using a motorized Potter-type glass/glass (0.045-0.065 mm clearance, 5 strokes) homogenizer (Braun) at 925 RPM. The suspension was then used as the bottom layer of a discontinuous Ficoll gradient upon which was sequentially layered 10 ml of 7.5% Ficoll and 10 ml of HB. The gradient was centrifuged (111,000 *g*, 30 min, 4 °C, swinging bucket SW-28 rotor). The synaptosomal layer that formed at the interface of the 12% and 7.5% Ficoll layers was collected with a Pasteur pipette and homogenized (motorized Potter-type, glass/glass, 0.045-0.065 mm clearance, 5 strokes, 925 RPM). The suspension was gently mixed with ≈ 25 ml of HB and centrifuged (28,150 *g*, 20 min, 4 °C, SS-34 rotor). The resulting pellet was collected and suspended in AB (0.2 ml/mg wet wt). This resulted in a synaptosomal suspension of approximately 0.2 mg protein/ml. The synaptosomes, which were stored on ice, were used for transport assays within a few hours of preparation.

Synaptosomal Transporter Activity:

The following protocol is based upon the method of Kuhar and Zarbin (1978). A 100 µl aliquot of the synaptosomal suspension was added to an assay tube and preincubated at 25 °C for 5 min. The assay was conveniently carried out in duplicate. Uptake was initiated by the addition of a 100 µl aliquot of AB containing [^{3}H]-D-aspartate (1-20 µM, final concentration). In those assays that evaluated inhibitor activity, the 100 µl aliquot of AB contained both the [^{3}H]-D-aspartate and potential inhibitors to ensure simultaneous addition. Following a 2 min incubation at 25 °C, the assay tubes were rapidly transferred to ice and quenched by the addition of 4 ml of ice cold AB. The synaptosomal suspension was quickly vacuum (≈ 20 mm Hg) filtered through GF/F (Whatman) glass micro-fiber filters. The assay tubes and filters were sequentially rinsed with an additional 4 ml of ice cold AB. The termination of the assay required less than 10 seconds to complete. The filters containing the synaptosomes were transferred to vials containing liquid scintillation fluid and allowed to dissolve for 24 hours. Retained radioactivity was quantified by liquid scintillation counting and corrections for nonspecific uptake (e.g., leakage, binding) were made by subtracting the amount of [^{3}H]-D-aspartate accumulated at 4 °C. Initial assays confirmed that uptake was both sodium-dependent, by utilizing sodium-free (choline-substituted) buffer, and linear with respect to time and protein content (Bridges et al., 1991). Kinetic constants of substrates and inhibitors were determined using this same procedure and by appropriately varying the concentrations of [^{3}H]-D-

aspartate and inhibitor with respect to K_m and K_i values (for review see: Christensen, 1975).

Measurement of Synaptosomal Efflux:

Synaptosomes were prepared as described above, except that the final suspension was made in a smaller volume of AB (0.04 ml/mg wet wt). A 9.9 ml aliquot of this suspension was transferred to an assay tube containing 100 µl of [^{3}H]-D-aspartate (2.5 µM, final concentration) and incubated at 25 °C for 15 min. The synaptosomal suspension was centrifuged (28,150 *g*, 20 min) to reisolate the [^{3}H]-D-aspartate-containing synaptosomes, which were then suspended in 5.0 ml of ice cold AB and maintained in an ice bath. The assay was conveniently carried out in duplicate. The exchange assays were initiated by the addition of 100 µl of the suspension to 2.9 ml of AB, which contained potential substrates and/or metabolic inhibitors and was pre-equilibrated to 37 °C. The assay was terminated 1-10 minutes later by rapidly quenching with 4 ml of ice cold AB followed by vacuum filtration (≈ 20 mm Hg) through GF/F glass fiber filters. The assay tubes and filters were sequentially rinsed with an additional 4 ml of ice cold AB. The filters containing the synaptosomes were transferred to vials containing liquid scintillation fluid and allowed to dissolve for 24 hours. Retained radioactivity was quantified by liquid scintillation counting and used to calculate changes in the rate of efflux of [^{3}H]-D-aspartate produced by the presence of the various compounds. The initial content of the synaptosomes ("0" time) was established by adding the 100 µl aliquot of synaptosomes to 2.9 ml of ice cold AB followed

immediately by filtering and rinsing. This value was determined at the beginning, middle, and end of each experiment to ensure that the amount of [^{3}H]-D-aspartate in the synaptosomes did not significantly change while being maintained in the ice bath. To assure that appropriate comparisons were made between experiments, it was verified that the synaptosomes initially contained similar amounts of [^{3}H]-D-aspartate, as internal content could have effected rates of efflux.

Substrates could be identified by their ability to increase the rate of efflux observed in AB alone. In contrast, nontransportable inhibitors could be identified by both their inability to increase the rate of efflux and their ability to competitively inhibit the heteroexchange of other substrates. In this respect, well characterized nontransportable inhibitors (e.g., dihydrokainate) could be used to inhibit substrate-induced [^{3}H]-D-aspartate efflux and verified that heteroexchange was occurring via the sodium-dependent transporters.

Oocyte Transport: (performed in collaboration with M.P. Kavanaugh, Vollum Institute)

Capped RNA was transcribed from linearized plasmids containing the coding regions of EAAT1-3 (Arriza et al., 1994). RNA (50 ng) was injected into stage V *Xenopus* oocytes and experiments were performed 2-6 days later. Current recordings were made with a two-microelectrode voltage clamp circuit. Electrodes contained 3 M KCl and had resistances of 100-500 kOhm. Oocytes were voltage clamped and continuously superfused with Ringer recording solution containing (in mM): NaCl, 98.5; $CaCl_2$, 1.8;

$MgCl_2$, 1.0; HEPES, 5 (pH 7.5). Glutamate and analogues were bath-applied by switching to a solution containing the compounds at the indicated concentrations. The concentration-dependence of the currents induced by the compounds were fitted by least squares to the equation $I=I_{max}([compound]/([compound]+K_m)$. K_m values were expressed as mean ± S.E.M. from fits to individual oocytes. Nontransportable inhibitors were co-applied with varying concentrations of L-glutamate, and Schild analysis (Arunlakshana and Schild, 1959) was performed to obtain estimates of inhibitor equilibrium dissociation constants.

Materials:

[^{3}H]-D-Aspartate was purchased from NEN (Boston, MA). L-Glutamate, D-aspartate, dihydrokainate, kainate, D,L-β-THA, iodoacetate, potassium cyanide, and Ficoll were obtained from Sigma (St. Louis, MO). *cis*-ACBD was purchased from Tocris (Ballwin, MO). 2,4-MPDC, L-*cis*-2,4-PDC, L-*trans*-2,4-PDC, L-*trans*-2,3-PDC, L-*cis*-2,3-PDC, D-*trans*-2,3-PDC, D-*cis*-2,3-PDC, *cis*-5-methyl-L-*trans*-2,3-PDC, L-*anti-endo*-3,4-MPDC, L-*anti-exo*-3,4-MPDC, L-*syn-exo*-3,4-MPDC and L-*trans*-2,3-ADC were synthesized as described (Bridges et al., 1991; Bridges et al., 1993; Bridges et al., 1994; Humphrey et al., 1994; Willis et al., 1997; Esslinger et al., 1998).

Chapter 3: Synaptosomal Pharmacology of Conformationally Constrained Analogues of L-Glutamate.

Introduction

Considering the important roles played by the EAA transporters in both normal synaptic transmission and in disease processes, it is not surprising that significant effort has been directed towards delineating the pharmacology of this system. Landmark studies focused on establishing the existence of a distinct system for glutamate uptake, and by utilizing EAA analogues and other acidic amino acids, these investigations began to define the basic pharmacophore of glutamate transport. A great advance towards this direction followed the development of conformationally constrained analogues of L-glutamate. Often these compounds were designed with the idea of embedding L-glutamate, L-aspartate, or portions thereof into a ring system and thus limiting the total number of conformations attainable by the molecule. This strategy led to the development and identification of uptake inhibitors such as L-*trans*-2,4-pyrrolidine dicarboxylate (L-*trans*-2,4-PDC), *cis*-1-aminocyclobutane-1,3-dicarboxylate (*cis*-ACBD), and (2S,3S,4R)-2-(carboxycyclopropyl)glycine (L-CCG-III). The success of these compounds as selective inhibitors of glutamate uptake led to the development of other potent, conformationally constrained inhibitors and thus the further refinement of the transporter pharmacophore. In this chapter, several novel series of analogues, including the 2,3-pyrrolidine dicarboxylates and 3,4-methanopyrrolidine dicarboxylates, were investigated for their activity at

the high-affinity glutamate transport system present in rat forebrain synaptosomes.

Results and Discussion

2,3-Pyrrolidine Dicarboxylates

Four stereoisomers of 2,3-pyrrolidine dicarboxylate (2,3-PDC) were synthesized by Rapoport-type alkylation of aspartic acid as described by (Humphrey et al., 1994). When L-*trans*-2,3-PDC, L-*cis*-2,3-PDC, D-*trans*-2,3-PDC, and D-*cis*-2,3-PDC were tested for the ability to inhibit the uptake of [^{3}H]-D-aspartate into forebrain synaptosomes, only the L-*trans* isomer proved effective (Table 3.1). Uptake of 5 μM D-aspartate was decreased to 24 ± 5% of control (i.e. absence of inhibitor and corrected for leakage and background, 0.99 ± 0.15 nmol/min/mg protein, n = 12 sets of duplicate assays) when L-*trans*-2,3-PDC was included in the assay at 250 μM. In contrast, when the L-*cis*, D-*trans*, and D-*cis* isomers were included at similar concentrations, they were found to be considerably less effective. L-*trans*-2,3-PDC decreased uptake to 67 ± 2% of control when the concentration of the inhibitor was reduced to 25 μM. The extent of inhibition produced by L-*trans*-2,3-PDC at 25 μM is comparable to that observed with several other known glutamate uptake inhibitors at this same concentration, including dihydrokainate (52 ± 4% of control), *cis*-5-Me-L-*trans*-2,3-PDC (80 ± 4% of control), L-*cis*-2,4-PDC (82 ± 2% of control), and *cis*-ACBD (88 ± 3% of control) (Table 3.1). A more detailed kinetic analysis of the action of L-*trans*-2,3-PDC is shown in Figure 3.1. The

representative Lineweaver-Burk plot demonstrates the activity of a competitive inhibitor, i.e., increasing concentrations of the inhibitor yielded increased values for $K_{m,app}$ with no change in V_{max}. The K_m and V_{max} values for the transport of D-aspartate were found to be 2.6 ± 0.3 μM and 2.3 ± 0.1 nmol/min/mg protein, respectively. A slope replot (see figure 3.1 inset) of the $K_{m,app}$ values yielded a K_i value of 23 ± 1 μM for this particular experiment, and the average of 4 such determinations resulted in an average K_i value of 33 ± 6 μM (Table 3.2).

3,4-Methanopyrrolidine Dicarboxylates

The three diastereomers of L-3,4-methanopyrrolidine dicarboxylate (L-MPDC) shown in Table 3.1 were prepared by treating the methyl ester of CBZ-L-dehydroproline with ethyldiazoacetate in the presence of rhodium acetate, as described previously (Bridges et al., 1994). L-*anti-endo*-3,4-MPDC, L-*anti-exo*-3,4-MPDC and L-*syn-exo*-3,4-MPDC were each tested for the ability to inhibit the high-affinity glutamate transporter. Of the three isomers, only L-*anti-endo*-3,4-MPDC was effective in inhibiting the uptake of [^{3}H]-D-aspartate into forebrain synaptosomes. When included in the assay at 250 μM, the transport of D-aspartate (5 μM) was reduced to a level that could not be differentiated from background. Uptake was reduced to 26 ± 3% of control when the concentration of L-*anti-endo*-3,4-MPDC was decreased to 25μM. The rate of uptake in the control experiments was 0.82 ± 0.17 nmol/min/mg protein (n=6). The extent of inhibition observed with L-*anti-endo*-3,4-MPDC at 25 μM is comparable to that produced by L-*trans*-2,4-PDC (19 ± 6% of control), D,L-β-THA (20 ± 4% of control), L-

aspartate (18 ± 3% of control) and L-glutamate (39 ± 3% of control) at this same concentration (Table 3.1). The results of more detailed studies of the inhibitory action of L-*anti-endo*-3,4-MPDC as a function of concentration are shown in Figure 3.2. The pattern of inhibition exhibited by the Lineweaver-Burke plot is consistent with that of a competitive inhibitor, and a replot of the $K_{m,app}$ values generated a K_i value of 2.5 ± 0.1 μM. The average of 3 similar determinations yielded a K_i value of 4.9 ± 2 μM (Table 3.2).

2,4-Methanopyrrolidine Dicarboxylates

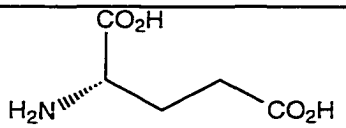
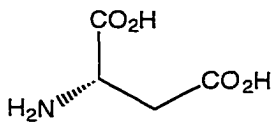
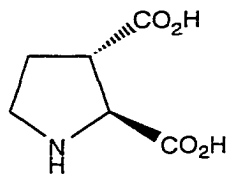
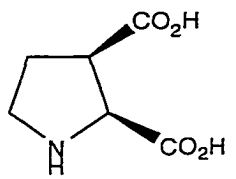
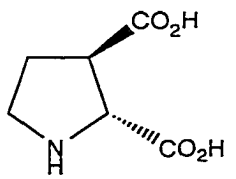
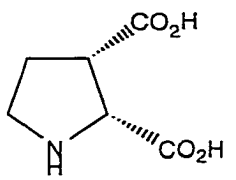
Intramolecular [2+2] photocyclization was used to prepare 2,4-methanopyrrolidine-2,4-dicarboxylate (2,4-MPDC) as described by (Esslinger et al., 1998). At 250 μM, 2,4-MPDC produced an almost complete inhibition of uptake of [^{3}H]-D-aspartate into forebrain synaptosomes (4 ± 0.1% of Control, i.e., 1.1 ± 0.2 nmol/min/mg protein, n = 16). Transport was reduced to 38 ± 1% of Control when the level of 2,4-MPDC was decreased to 25 μM. This inhibition is comparable to that produced by similar amounts of L-*trans*-2,4-PDC, L-*anti-endo*-3,4-MPDC, D,L-β-THA or L-glutamate (Table 1). A representative Lineweaver-Burk plot and replot of $K_{m,app}$ vs inhibitor concentration (inset) for 2,4-MPDC are depicted in Figure 3.3. The pattern of inhibition observed in this analysis was consistent with competitive inhibition and yielded values of: K_m = 3.8 (D-aspartate), K_i = 5.9 μM, and a V_{max} = 3.3 nmol/min/mg protein. Eight such analyses yielded an average K_i = 6.8 ± 3 μM (Table 3.2).

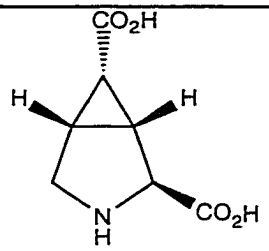
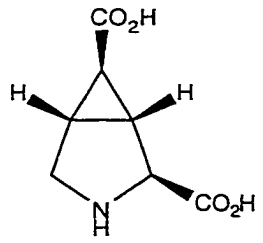
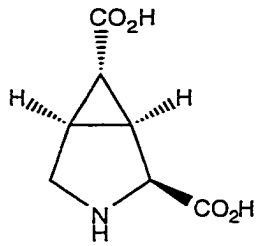
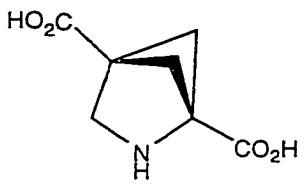
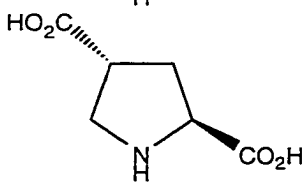
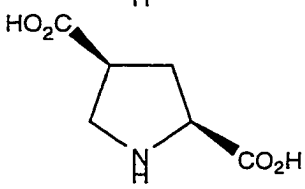
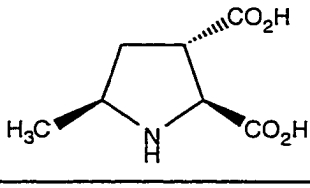
On the basis of the K_i values listed in Table 3.2, the competitive inhibitors of high-affinity glutamate transport could be roughly divided into three categories. Potent analogues could be considered those similar in potency to L-glutamate and L-aspartate (K_i = 1.5 - 7 μM) and include L-*trans*-2,4-PDC, D,L-β-THA, L-*anti-endo*-3,4-MPDC, and 2,4-MPDC. Moderately potent analogues were more comparable in potency to the well known inhibitor dihydrokainate ($K_i \approx$ 30 μM) and include *cis*-ACBD, L-*trans*-2,3-PDC, and *cis*-5-Me-L-*trans*-2,3-PDC. Three of the analogues, kainate, L-*cis*-2,4-PDC and L-*trans*-2,3-ADC, were also competitive inhibitors but were markedly less potent than dihydrokainate, exhibiting K_i values around 70 μM and could thus be considered weak inhibitors.

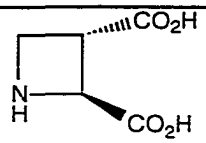
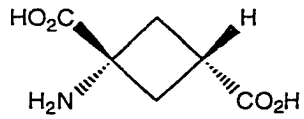
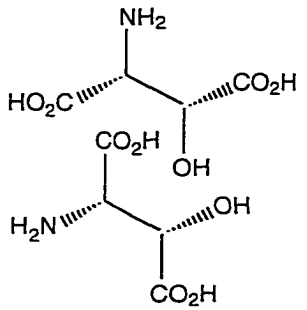
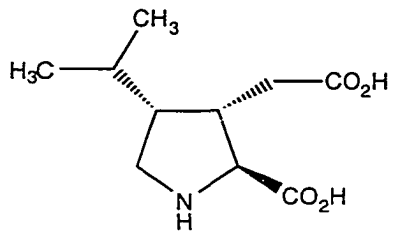
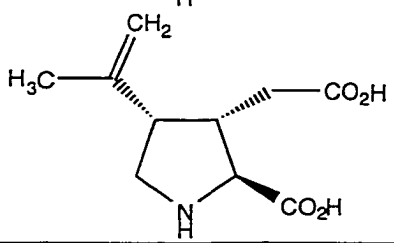
The constrained analogues, as a result of limited structural flexibility, were highly useful in determining the arrangement of the functional groups required for binding to the transporter protein. Energy minimizations of L-glutamate, L-*trans*-2,3-PDC, L-*trans*-2,4-PDC, L-*anti-endo*-3,4-MPDC and 2,4-MPDC were carried out using SYBYL molecular modeling software (Tripos, St. Louis, MO) on a Silicon Graphics Indigo 2 workstation. Molecular superimposition of the analogues was then conducted using a three point best fit of the amine functionality, α-carboxylate carbon and distal carboxylate carbon of the minimized structures. Pairwise comparisons of the three selected atoms were made between the conformations of L-glutamate and each of the analogues, and yielded root mean square (RMS) deviations of 0.346 Å (L-*trans*-2,3-PDC), 0.391 Å (L-*trans*-2,4-PDC), 0.024 Å (L-*anti-endo*-3,4-MPDC), and 0.132 Å (2,4-MPDC). This procedure is identical to that previously used to define the transporter pharmacophore (Bridges et al., 1991; Bridges et al., 1993;

Chamberlin et al., 1998), and resulted in structures with a high degree of overlap and similar functional group arrangements (Figure 3.4). From this point, additional insight into the transporter pharmacophore was gained by considering the space occupied by the rings of the constrained analogues. This volume represents steric bulk not occupied by the acyclic backbone of L-glutamate but that is tolerated by the transporter binding site. For example, the volumes occupied by C4-C5 of L-*trans*-2,3-PDC, C5 of L-*trans*-2,4-PDC, C4-C5 of L-*anti-endo*-3,4-MPDC, and the methano-bridge of 2,4-MPDC may indicate regions of the binding site that can accommodate the excess steric bulk of a constrained inhibitor. These observations will appear more significant in the following chapter on the differentiation of substrate from nonsubstrate inhibitors, and will no doubt provide further guidance for the development of other potent and selective inhibitors of the high-affinity glutamate transporters.

Table 3.1 Inhibitory activities of glutamate and aspartate analogues on the synaptosomal uptake of [^{3}H]D-aspartate.

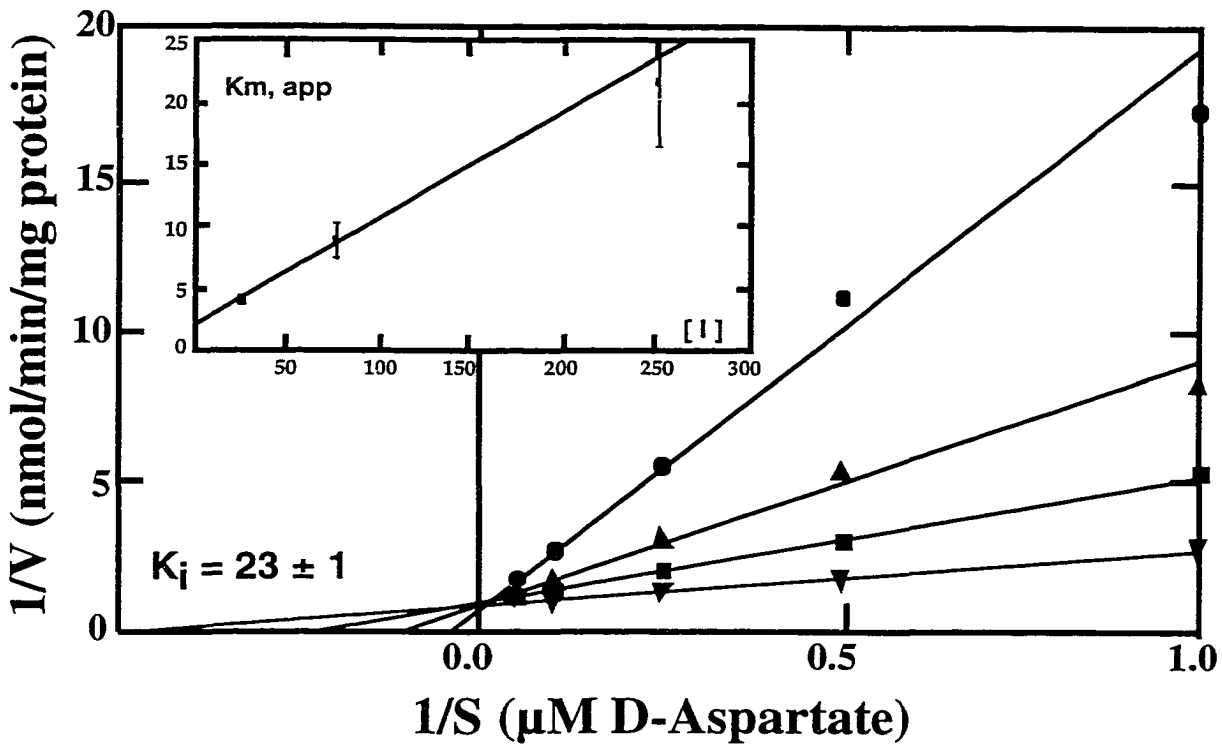
Compound	Structure	Uptake of 5μM [^{3}H]D-aspartate (% of Control) 250 μM	25 μM
L-Glutamate		2 ± 2	39 ± 3
L-Aspartate		0 ± 0	18 ± 3
L-*trans*-2,3-PDC		24 ± 5	67 ± 2
L-*cis*-2,3-PDC		81 ± 8	n.d.
D-*trans*-2,3-PDC		108 ± 8	n.d.
D-*cis*-2,3-PDC		107 ± 7	n.d.

Compound	Structure	Uptake of 5μM [^{3}H]D-aspartate (% of Control) 250 μM	25 μM
L-*anti-endo*-3,4-MPDC		0 ± 0	26 ± 3
L-*anti-exo*-3,4-MPDC		104 ± 6	n.d.
L-*syn-exo*-3,4-MPDC		111 ± 8	n.d.
2,4-MPDC		4 ± 0.1	38 ± 1
L-*trans*-2,4-PDC		0 ± 0	19 ± 6
L-*cis*-2,4-PDC		36 ± 4	82 ± 2
cis-5-Me-L-*trans*-2,3-PDC		30 ± 2	80 ± 4

Structure	Compound	Uptake of 5μM [^{3}H]D-aspartate (% of Control) 250 μM	25 μM
L-*trans*-2,3-ADC		53 ± 5	96 ± 6
cis-ACBD		26 ± 1	88 ± 3
D,L-β-THA		0 ± 0	20 ± 4
Dihydrokainate		25 ± 2	52 ± 4
Kainate		39 ± 4	n.d.

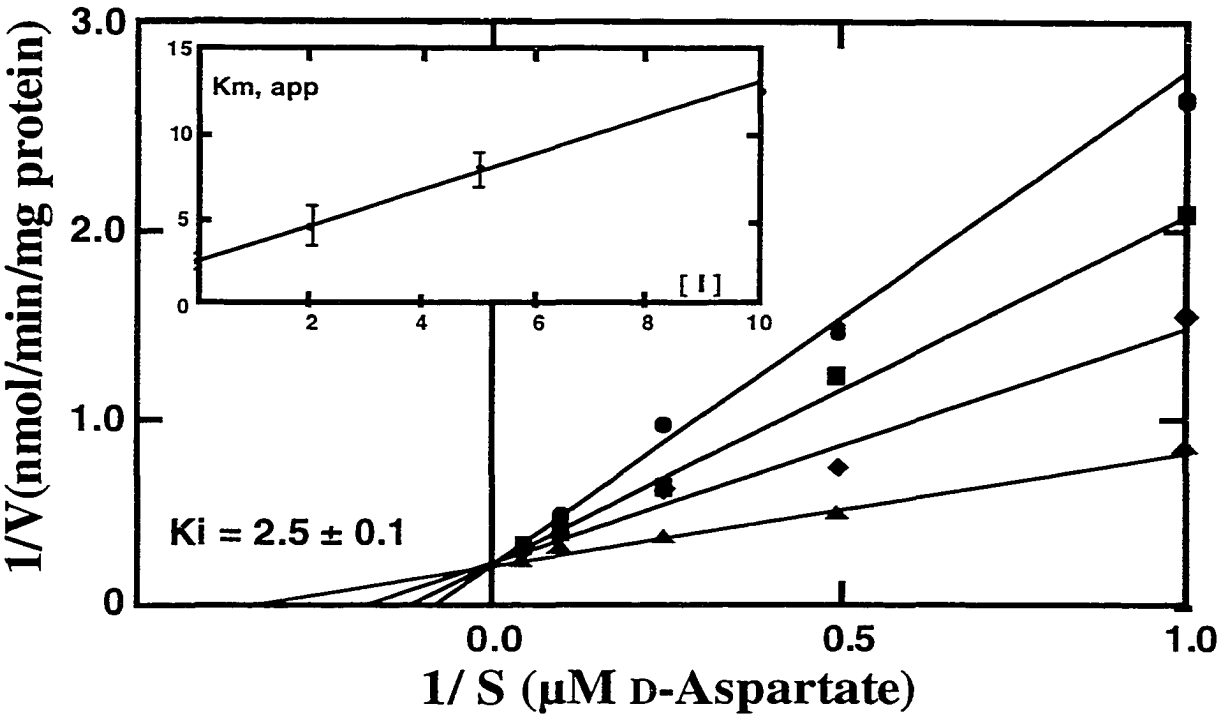
Synaptosomal uptake of [^{3}H]-D-aspartate is reported as mean % of control ± standard deviation. The data for each compound was based on at least three duplicate determinations each from separate synaptosomal preparations. Average control uptake of [^{3}H]-D-aspartate in the synaptosomal preparations was 0.97 nmol/min/mg protein. The assays were performed as described in Methods and Materials and have been corrected for nonspecific uptake and leakage. n.d. denotes not detected.

Figure 3.1 Competitive inhibition of [^{3}H]-D-aspartate uptake into synaptosomes by L-*trans*-2,3-PDC.



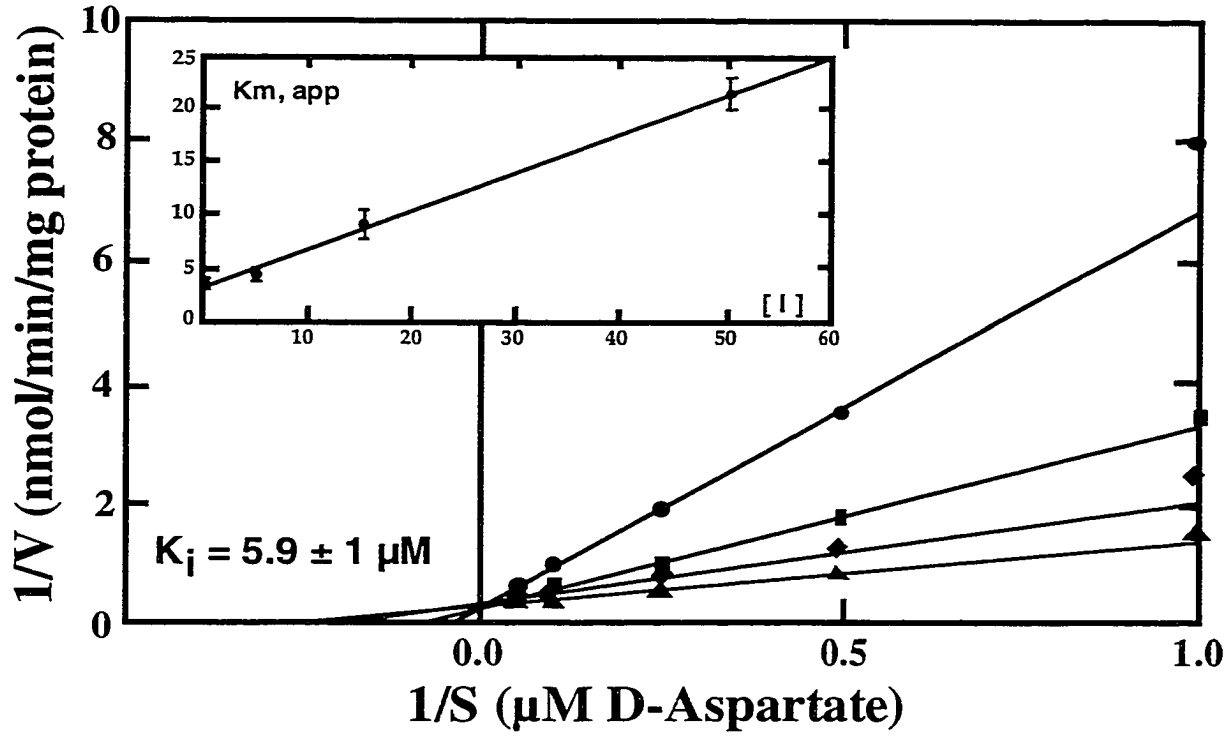
Representative Lineweaver-Burk plot demonstrating the competitive inhibition of [^{3}H]-D-aspartate uptake into forebrain synaptosomes by L-*trans*-2,3-PDC. The inset shows a replot of $K_{m,app}$ vs [L-*trans*-2,3-PDC]. The plots shown were generated using the k-cat kinetic program (BioMetallics Inc., Princeton, NJ) with weighting based on constant relative error, and yielded values of: K_m = 2.6 ± 0.3 μM (D-aspartate), V_{max} = 2.3 ± 0.1 nmol/min/mg protein, and K_i = 23 ± 1.

Figure 3.2 Competitive inhibition of [^{3}H]-D-aspartate uptake into synaptosomes by L-*anti-endo*-3,4-MPDC.



Representative Lineweaver-Burk plot demonstrating the competitive inhibition of [^{3}H]-D-aspartate uptake into forebrain synaptosomes by L-*anti-endo*-3,4-MPDC. The inset shows a replot of $K_{m,app}$ vs [L-*anti-endo*-3,4-MPDC]. The plots shown were generated using the k-cat kinetic program (BioMetallics Inc., Princeton, NJ) with weighting based on constant relative error, and yielded values of: K_m = 2.7 ± 0.3 µM (D-aspartate), V_{max} = 1.7 ± 0.1 nmol/min/mg protein, and K_i =2.5 ± 0.1.

Figure 3.3 Competitive inhibition of [^{3}H]-D-aspartate uptake into synaptosomes by 2,4-MPDC.



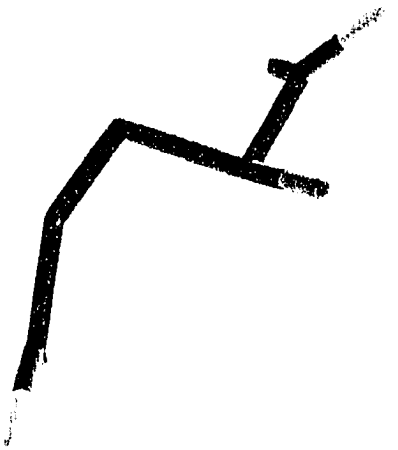
Representative Lineweaver-Burk plot demonstrating the competitive inhibition of [^{3}H]-D-aspartate uptake into forebrain synaptosomes by 2,4-MPDC. The inset shows a replot of $K_{m,app}$ vs [2,4-MPDC]. The plots shown were generated using the k-cat kinetic program (BioMetallics Inc., Princeton, NJ) with weighting based on constant relative error, and yielded values of: K_m = 3.8 ± 0.5 µM (D-aspartate), V_{max} = 3.3 ± 0.2 nmol/min/mg protein, and K_i = 5.9 ± 1.

Table 3.2 K_i values for inhibitors of synaptosomal [^{3}H]D-aspartate uptake.

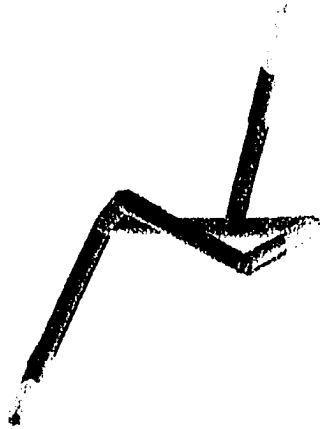
Compound	K_i (μM)
L-Glutamate	4.9 ± 1
L-Aspartate	1.7 ± 0.6
L-*trans*-2,4-PDC	1.5 ± 0.5
D,L-β-THA	2.0 ± 1
L-*anti-endo*-3,4-MPDC	4.9 ± 2
2,4-MPDC	6.8 ± 3
Dihydrokainate	28 ± 2
cis-ACBD	30 ± 2
L-*trans*-2,3-PDC	33 ± 6
cis-5-Me-L-*trans*-2,3-PDC	37 ± 6
Kainate	59 ± 6
L-*cis*-2,4-PDC	66 ± 6
L-*trans*-2,3-ADC	78 ± 6

Table 3.2: The values reported in this table represent the average K_i values ± standard deviation as determined in at least 3 separate Lineweaver-Burk analyses (see representative plots, Figures 3.1 - 3.3) of the inhibiton of the Na^+-dependent uptake of [^{3}H]-D-aspartate into synaptosomes. The plots were prepared using the k-cat kinetic program (BioMetallics Inc., Princeton NJ) with weighting based on constant relative error. K_i values were estimated on the basis of a replot of $K_{m, app}$ values.

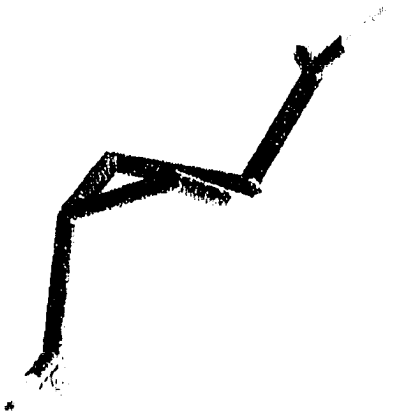
Figure 3.4 Proposed Transporter Binding Conformations of Glutamate Analogues



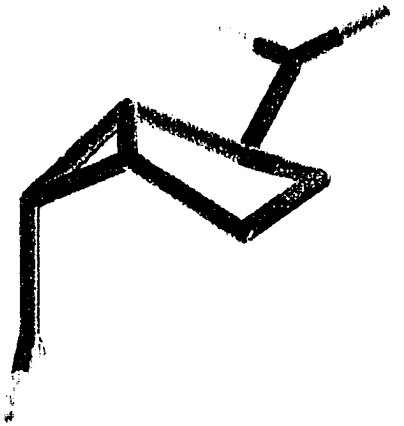
L-Glutamate



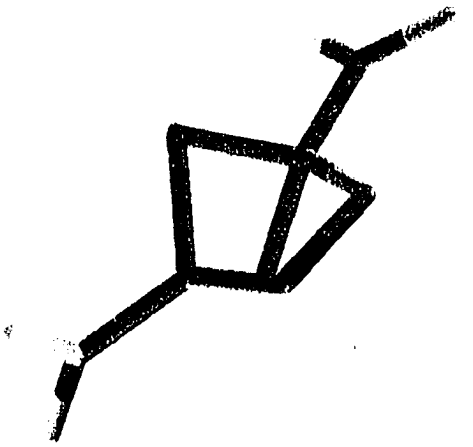
L-*trans*-2,3-PDC



L-*trans*-2,4-PDC



L-*anti-endo*-3,4-MPDC



2,4-MPDC

Figure 3.4: Molecular modeling of the proposed glutamate transporter binding conformations of L-glutamate, L-*trans*-2,3-PDC, L-*trans*-2,4-PDC, L-*anti-endo*-3,4-MPDC, and 2,4-MPDC was performed on a Silicon Graphics workstation using SYBYL modeling software (Tripos, St. Louis, MO). All molecules are minimized conformations with hydrogens omitted for clarity. Molecular comparisons were achieved using a three point fit of the α-carboxylate carbon (darkest gray represents carbon backbone), amino group (medium gray) and distal carboxylate carbon (lightest gray depicts carboxylate moieties), and yielded an average RMS deviation of 0.32 ± 0.12 Å).

Chapter 4: Differentiation of Substrate and Nonsubstrate Inhibitors of Synaptosomal Transport

Introduction

Much of our understanding of the contribution of sodium-dependent, high-affinity glutamate transporters to EAA physiology and pathology has emerged as a consequence of the ability to pharmacologically manipulate transporter activity. The majority of studies characterizing glutamate transport inhibitors, however, have often neglected to address whether or not the selective blockers are also transported, i.e., whether they also act as substrates for the transporter. While this omission has been partially addressed by electrophysiologically recording substrate-induced currents in voltage-clamped oocytes expressing cloned transporters (Arriza et al., 1994), it remains a significant obstacle in more complex physiological preparations obtained directly from CNS tissues (e.g., synaptosomes, tissue slices, primary cell culture). In the present study, we test the hypothesis that transporter-mediated heteroexchange can be readily used in a classical synaptosomal preparation to distinguish transportable from non-transportable inhibitors.

A library of conformationally constrained glutamate analogues (see Table 3.1 and Table 4.1) was brought to bear upon the synaptosomal glutamate transport systems for the purpose of addressing two key issues:

the molecular differentiation of substrate and nonsubstrate inhibitors, and the comparison of this extensively used physiological preparation to the activities of the cloned glutamate transporters. It was demonstrated that the process of transporter-mediated heteroexchange, in which an externally applied substrate stimulates the efflux of an internal (radiolabeled) substrate that had been previously sequestered in the synaptosomes, can be exploited to rapidly differentiate substrates from nontransportable inhibitors. A number of potent inhibitors were identified that shared similar positionings of their functional groups, yet exhibited markedly different capacities to serve as substrates for the transporter. These analogues were then used in molecular modeling studies to refine our pharmacophore model of the substrate binding site on the transporter. Interestingly, the resulting pharmacological profile of substrates and nontransportable inhibitors also demonstrated that the synaptosomal preparation exhibited the greatest correspondence with EAAT2, a transporter believed to be primarily of glial origin (for review see: Gegelashvili and Schousboe, 1997).

Results and Discussion

The conformationally constrained competitive inhibitors listed in Table 4.1 were tested as potential substrates by assaying their ability to

exchange with, and stimulate the efflux of, a radiolabeled substrate from inside the synaptosomes. Thus, synaptosomes were incubated with [^{3}H]-D-aspartate, reisolated by ultracentrifugation, resuspended ([^{3}H]-D-aspartate content, 1152±65 pmol/mg protein) and then diluted 30-fold into assay buffer at 37°C in the presence and absence of potential substrates. Analogues were tested at 100 μM and, to ensure comparable levels of occupancy of the transporter binding sites, at a concentration approximately 10-fold greater than the K_i values with which they inhibited uptake. The extent of efflux was quantified over a two min period. The values were corrected for the efflux which occurred in the absence of inhibitor (228 ± 8 pmol /mg protein/2 min) and are summarized in Table 4.1. The analogues exhibited a wide range of substrate activities that did not necessarily correlate with the ability of the compounds to bind to the transporter. For example, L-glutamate and L-*anti-endo*-3,4-MPDC were equally effective at inhibiting uptake (K_i values ≈ 5 μM), although L-*anti-endo*-3,4-MPDC exhibited only a third of the substrate activity of L-glutamate. In contrast, 2,4-MPDC (K_i = 6.8 μM) proved to be almost equipotent with L-glutamate in stimulating exchange, indicating that conformational restriction does not necessarily convey a low substrate activity. Interestingly, *cis*-ACBD and L-*cis*-2,4-PDC (K_i = 30 μM and 66 μM, respectively) also produced a marked increase in the efflux of [^{3}H]-D-aspartate when present at concentrations sufficient enough to ensure high

levels of binding. These results suggest that even moderate-to-weak inhibitors can be effectively translocated once bound to the transporter substrate site. Although kainate, dihydrokainate, L-*trans*-2,3-PDC, and *cis*-5-Me-L-*trans*-2,3-PDC were identified as competitive inhibitors, these compounds did not produce levels of efflux that were significantly different from what was observed in the absence of inhibitor. Additionally, when included in the efflux assay in combination with L-glutamate, both dihydrokainate and L-*trans*-2,3-PDC effectively attenuated glutamate-mediated exchange, consistent with the action of nontransportable inhibitors. Similar results were observed in more detailed studies that examined the time course with which the substrates and nonsubstrate inhibitors altered the efflux of [^{3}H]-D-aspartate from the synaptosomes. As illustrated in Figure 4.1A, little or no efflux of radiolabel was observed when the synaptosomes were maintained at 4°C (≤ 40 min). However, when the synaptosomes were diluted into assay buffer at 37°C in the presence of L-glutamate (10 μM), the resulting efflux of [^{3}H]-D-aspartate was more than 2-fold greater than that observed in assay buffer alone. On the other hand, L-*trans*-2,3-PDC (300 μM) not only failed to stimulate heteroexchange by itself, but it also reduced the level of glutamate-mediated heteroexchange when it was combined in the assay with this substrate (Figure 4.1A).

The specificity of the exchange process, which is critical to evaluating the utility of this approach for identifying glutamate transporter substrates and inhibitors, was evaluated by conducting a parallel series of experiments with GABA. As shown in Figure 4.1A and Figure 4.2, the efflux of [^{3}H]-D-aspartate was unaffected by the inclusion of GABA (50 μM) in the dilution buffer. Conversely (Figure 4.1B and Figure 4.2), the efflux of radiolabel from synaptosomes containing [^{3}H]-GABA (3887±263 pmol/mg protein) was markedly enhanced by the presence of GABA (50 μM, i.e., ≈ 10 x Ki) in the assay buffer, but not by L-glutamate (10 μM). Similarly, GABA-mediated homoexchange was not inhibited by L-*trans*-2,3-PDC (300 μM). Additional experiments demonstrated that the efflux of [^{3}H]-D-aspartate or [^{3}H]-GABA from the synaptosomes was not altered by the presence of NMDA (100 μM), kainate (100 μM), AMPA (100 μM), or *trans*-ACPD (100 μM), ruling out the potential contribution of EAA receptors to the exchange process (Figure 4.2).

The validity of this approach rests on the pretense that the observed synaptosomal efflux of [^{3}H]-D-aspartate that occurs as a result of an externally applied compound represents transporter-mediated exchange of internal for external substrate. That this was indeed occurring in the present experiments is supported by the demonstration that: *i*) efflux was stimulated in a substrate-specific manner (e.g., L-glutamate and L-aspartate, but not GABA, are active), *ii*) efflux was unaffected by the EAA

receptor agonists NMDA, KA, AMPA or *trans*-ACPD, and *iii*) substrate-induced increases in efflux could be attenuated by analogues identified electrophysiologically and biochemically as nontransportable inhibitors (e.g., dihydrokainate, L-*trans*-2,3-PDC) (Johnston et al., 1979; Arriza et al., 1994). Thus, compounds could be selectively tested for two distinct steps in the synaptosomal uptake process: competitive inhibition of uptake as an indicator of binding and the stimulation of heteroexchange as a measure of substrate activity.

Comparisons of these two properties within the context of our library of conformationally constrained analogues suggest that the ability of a compound to bind to the transporter does not necessarily dictate its activity as a substrate. This is readily exemplified by L-glutamate, L-*anti-endo*-3,4-MPDC, and 2,4-MPDC, each of which competitively inhibited uptake with comparable K_i values ($\approx$ 5 - 7 μM). Thus, while these compounds appear equal in their ability to bind to the transporter, 2,4-MPDC and L-*anti-endo*-3,4-MPDC were translocated only about 80% and 35% as effectively as L-glutamate, respectively. Such differences in substrate activity were not limited to just those compounds exhibiting K_i values similar to L-glutamate. For example, L-aspartate, L-*trans*-2,4-PDC and D,L-β-THA each inhibited the uptake of [^{3}H]-D-aspartate more potently than L-glutamate (e.g., K_i values = 1.5 - 2 μM), yet varied greatly in their ability to stimulate exchange (e.g., 80%, 60% and 47% the activity of L-

glutamate, respectively). Even more surprising than the substrate activity of these potent inhibitors was the finding that the moderate-to-weak inhibitors ($K_i \approx$ 30-70 μM) also included both substrates (*cis*-ACBD, L-*cis*-2,4-PDC, L-*trans*-2,3-ADC) and nonsubstrate inhibitors (e.g., *cis*-5-Me-L-*trans*-2,3-PDC, L-*trans*-2,3-PDC, dihydrokainate and kainate). Thus, while a compound like L-*cis*-2,4-PDC (K_i = 66 μM) may be considered inferior in its ability to initially bind to the substrate site of the transporter, once it occupies this site, it can be translocated as effectively as substrates such as L-aspartate. These findings are also consistent with previous demonstrations that L-*trans*-2,4-PDC and *cis*-ACBD can stimulate the efflux of [^{3}H]-D-aspartate from cultured cerebellar granule cells (Griffiths et al., 1994). Likewise, L-*trans*-2,4-PDC has been shown to exchange with [^{3}H]-L-glutamate in reconstituted liposomes containing glutamate transporters (Volterra et al., 1996).

While numerous chemical properties may influence whether or not a compound that binds to the transport protein is also translocated by the uptake system, the present library of analogues demonstrates that conformational restriction does not inherently differentiate transportable from nontransportable inhibitors. For example, the conformational rigidity produced through the incorporation of distinct pyrrolidine-containing, bicyclic ring systems in 2,4-MPDC and L-*anti-endo*-3,4-MPDC resulted in a similar positioning of their functional groups and

comparable K_i values as competitive inhibitors, yet 2,4-MPDC proved to be a very good substrate, while L-*anti-endo*-3,4-MPDC did not. Indeed, in a previous study with *Xenopus* oocytes, L-*anti-endo*-3,4-MPDC was found to be a nontransportable inhibitor (K_i = 1.6 μM) at EAAT-2 (Esslinger et al., 1998). Advantageously, the identification of representative transportable and nontransportable inhibitors that are conformationally rigid allows pharmacophore modeling to be extended beyond the process of binding and toward an initial delineation of the underlying chemical properties that influence substrate activity. To accomplish this, energy-minimized conformations of L-glutamate, and representative substrates (2,4-MPDC, L-*trans*-2,4-PDC and *cis*-ACBD) and nontransportable inhibitors (L-*trans*-2,3-PDC and L-*anti-endo*-3,4-MPDC) were first identified and then systematically overlaid using a Silicon Graphics workstation and SYBYL modeling software (Tripos, St. Louis).

The spatial positionings of the two negatively charged carboxylate functionalities (designated in red in Figure 4.5) and the positively charged ammonium functionality (designated in purple in Figure 4.5) of each analogue were compared in a three-point best fit analysis to identify conformers exhibiting the greatest degree of overlap. This approach is identical to that used previously to characterize the glutamate transporter binding site pharmacophore (Bridges et al., 1993; Chamberlin et al., 1998; Esslinger et al., 1998). As all substrates must first bind, the same conformer of glutamate was used as a starting point in both models. As shown in

Figure 4.5A, the substrates (L-*trans*-2,4-PDC (red), 2,4-MPDC (yellow), and *cis*-ACBD (white)) exhibited a high degree of overlap (e.g., RMS deviation of 0.42 ± 0.17 Å) with the minimized L-glutamate (green) conformation of the currently proposed pharmacophore. Similarly, the inhibitors identified as non-substrates (L-*trans*-2,3-PDC (orange) and L-*anti-endo*-3,4-MPDC (light blue)) also overlaid well with this conformation of L-glutamate (green), as shown in Figure 4.5B. When the overlays of both substrates and non-substrate inhibitors were compared (Figure 4.5C), it was noticed that while the functional group positioning was very similar (e.g., RMS deviation of 0.32 ± 0.12 Å), regions of excess steric volume associated with the carbon backbones of the nonsubstrates could be identified that were distinct relative to the space occupied by the backbones of the substrates. These regions (depicted in yellow framework in Figure 4.5D) become more obvious when molecular volumes of the substrates (Figure 4.5A) are subtracted from those of the nonsubstrates (Figure 4.5B). We suggest that owing to the rigidity of the compounds, it is likely that steric excess in these specific positions represents a determining, or at least predictive, factor in whether or not the analogues can be translocated. Thus, while the similar positionings of the charged functionalities allows binding to occur, the excess volume occupied by the backbones of those analogues identified as nontransportable inhibitors may sterically interact in an unfavorable manner with specific domains on the transporter protein that participate in translocating substrates once they are bound.

The actions of the analogues were also examined in *Xenopus* oocytes expressing cloned human glutamate transporters (performed in collaboration with M.P. Kavanaugh, Vollum Institute). Owing to the electrogenic nature of the translocation of L-glutamate and associated counter ions by the EAATs, substrates could be readily differentiated from nonsubstrates based upon the ability to produce an electrical current (Arriza et al., 1994). When 2,4-MPDC was applied to voltage-clamped oocytes expressing EAAT2, this analogue induced currents in a dose-dependent manner (Figure 4.3A) that yielded a K_m value of 45 μM. This analogue proved to be an excellent substrate of EAAT2, as it exhibited an I_{max} value 15% greater than that of L-glutamate itself (Table 4.2, (Esslinger et al., 1998)). While 2,4-MPDC also proved to be a substrate of both EAAT1 and EAAT3, in these instances it exhibited K_m values about two-fold greater than those observed at EAAT2 and I_{max} values of only about half those of L-glutamate (Table 4.2).

In contrast to the actions of 2,4-MPDC, application of 30 μM L-*trans*-2,3-PDC to oocytes expressing EAAT2 did not induce a current (Figure 4.3B). However, when co-applied with 30 μM L-glutamate, this analogue significantly reduced the substrate-induced currents normally observed with L-glutamate alone. Schild analysis of this inhibition was consistent with a competitive mechanism and yielded a K_d of 16 μM (Figure 4.3C). Furthermore, L-*trans*-2,3-PDC did not induce any currents in oocytes

expressing EAAT1 or EAAT3 (K_d>300 μM), and was ineffective at inhibiting glutamate-induced currents at these subtypes.

The electrophysiological characterization of transport into oocytes expressing human EAAT1, EAAT2 or EAAT3 provided an alternative approach to corroborate our conclusions regarding substrate activity as determined by heteroexchange, as well as a strategy to compare the pharmacological profile of synaptosomal uptake to that of the individual transporter subtypes. Using a select series of compounds that included both transportable and nontransportable inhibitors, we found a good correspondence between the ability of an analogue to stimulate the synaptosomal efflux of [^{3}H]-D-aspartate and its ability to induce substrate-mediated currents in the oocytes, particularly those expressing EAAT2 (Figure 4.4). At one extreme, 2,4-MPDC exhibited an I_{max} in EAAT2-expressing oocytes greater than that of L-glutamate itself and proved to be one of the most effective analogues in the synaptosomal exchange assays (Table 4.2). At the other extreme, L-*trans*-2,3-PDC (and dihydrokainate) was found to be inactive at either producing currents in these same oocytes or stimulating the efflux of [^{3}H]-D-aspartate from the synaptosomes (Table 4.2), yet was capable of competitively blocking the normal glutamate-mediated response in both systems. In the instance of the analogues that were identified as partial substrates in the exchange assays (e.g., L-*trans*-2,4-PDC and D,L-β-THA), previous studies have

demonstrated that these same compounds produce intermediate I_{max} values in oocytes expressing EAAT1, EAAT2, and EAAT3 (Arriza et al., 1994). It is 2,4-MPDC, L-*trans*-2,3-PDC, and dihydrokainate, however, that provided the most compelling evidence linking synaptosomal pharmacology with EAAT2, particularly within the context of substrate activity (Figure 4.4, Table 4.2). While comparisons of relative I_{max} values and efflux rates are admittedly qualitative in nature, L-*trans*-2,3-PDC and dihydrokainate were essentially inactive at EAAT1 and EAAT3, yet effective nontransportable inhibitors of both EAAT2 and the synaptosomal system. Another nontransportable inhibitor of EAAT2, L-*anti-endo*-3,4-MPDC (Esslinger et al., 1998), produced an efflux rate that was only a third of that produced by L-glutamate. 2,4-MPDC, on the other hand, proved to be an excellent substrate of both EAAT2 and the synaptosomes, yet produced I_{max} values at EAAT1 and EAAT3 that were only about half those of L-glutamate.

When similar attempts were made to compare the kinetic constants in the two systems, some difficulties arose because the recordings of the substrate-induced currents in the EAATs typically yielded K_m values, while the synaptosomal inhibitory assays resulted in K_i constants. This complication was avoided, however, in the instance of the nontransportable inhibitors, as both approaches yielded K_i values. In the present study we found that the K_i values for L-*trans*-2,3-PDC and dihydrokainate very similar to one another within each system, but about

3-fold less in the oocytes than in the synaptosomes. Such a relationship is consistent with recent studies demonstrating that dihydrokainate, kainate, and D,L-*threo*-β-benzyloxyaspartate (TBOA), all of which are non-transportable inhibitors at EAAT2, also exhibited lower K_i values when characterized in oocytes as compared to mammalian cells expressing the transporters (Arriza et al., 1994; Shimamoto et al., 1998).

The conclusion that EAAT2 corresponds most closely with the synaptosomal system is quite interesting in view of the general presumption that EAAT2 (rat homologue GLT-1), is thought to be chiefly expressed in astrocytes (for review see: Gegelashvili and Schousboe, 1998). The findings suggest then that either the synaptosomal preparation contains glial fragments possessing functional transporters, or that a separate yet unidentified glutamate transporter subtype exists in neurons that shares a similar pharmacology with EAAT2. While the latter cannot be ruled out, numerous reports indicate both the heterogeneous nature of synaptosomes (Henn et al., 1976; Nakamura et al., 1993) and that the predominant transport activity in this preparation is "EAAT2-like" in its pharmacology (Dowd et al., 1996; Gegelashvili and Schousboe, 1998). Significantly, it has also been demonstrated that synaptosomes prepared from mice deficient in GLT-1 exhibit a marked loss in the ability to transport L-glutamate (Tanaka et al., 1997). Regardless of the exact subtype(s) present in the synaptosomes, our data clearly indicate that within the context of the known transporter subtypes, the proposed

pharmacophore (as well as the synaptosomal assay system) should be considered as a working model for the EAAT2 subtype. In this regard, preliminary modeling studies indicate that more recently identified inhibitors of EAAT2 (e.g., T-BOA, (±)-*threo*-3-methylglutamate, 2S,4R-4-methylglutamate, and (±)-4-methyleneglutamate) (Vandenberg et al., 1997; Shimamoto et al., 1998), also fit within the proposed pharmacophore. Not only was there significant functional group overlap in each case, but the modifications to the glutamate/aspartate backbone present in those compounds identified as non-transportable inhibitors of EAAT2 (e.g., T-BOA, (±)-*threo*-3-methylglutamate, (2S,4R)-4-methylglutamate (Vandenberg et al., 1997; Shimamoto et al., 1998), overlapped with those regions depicted in the model (Figure 4.5) as tolerated for binding but not translocation. As these models are refined for each transporter subtype, they should prove valuable in the design of more selective substrates and nontransportable inhibitors, as well as identify important functional domains that must be incorporated into evolving structural models of the transporters.

Table 4.1: K_i values and Exchange Rates for Transport Inhibitors

Compound	K_i (μM)	Concentration (μM)	Exchange Rate (pmol/mg protein/2 min)
L-Glutamate	4.9 ± 1	100	362 ± 12
		50	344 ± 16
		10	316 ± 22
2,4-MPDC	6.8 ± 3	100	282 ± 28
		75	280 ± 30
L-Aspartate	1.7 ± 0.6	100	278 ± 16
		20	266 ± 22
cis-ACBD	30 ± 2	100	266 ± 4
		300	270 ± 8
L-*cis*-2,4-PDC	66 ± 6	100	206 ± 16
		500	260 ± 22
L-*trans*-2,4-PDC	1.5 ± 0.5	100	250 ± 28
		15	208 ± 28
D,L-β-THA	2.0 ± 1	100	172 ± 24
		20	162 ± 16

Compound	K_i (μM)	Concentration (μM)	Exchange Rate (pmol/mg protein/2 min)
L-*trans*-2,3-ADC	78 ± 6	100	146 ± 14
		800	156 ± 26
L-*anti-endo*-3,4-MPDC	4.9 ± 2	100	120 ± 20
		50	120 ± 18
Kainate	59 ± 6	100	0 ± 1.5
		600	0 ± 0
cis-5-Me-L-*trans*-2,3-PDC	37 ± 6	100	3 ± 6
		350	0 ± 0
Dihydrokainate	28 ± 2	100	0 ± 3
		300	11 ± 6
Dihydrokainate + L-Glutamate		300 10	60 ± 12
L-*trans*-2,3-PDC	33 ± 6	100	3 ± 6
		300	19 ± 6
L-*trans*-2,3-PDC + L-Glutamate		300 10	76 ± 16

Table 4.1: K_i values in this table are reported as the mean ± standard deviation of at least 3 separate Lineweaver-Burk analyses which were carried out as described in Chapter 3. The synaptosomal efflux of [^{3}H]-D-aspartate induced by the inhibitors was quantified over a 2 min period at 37°C and was corrected for the efflux that occurred in the absence of added compound (228 ± 8 pmol/mg protein/2 min). Inhibitors were tested both at 100 μM and, to ensure comparable levels of transporter binding, at concentrations approximately 10 times greater than the K_i values for which they inhibited [^{3}H]-D-aspartate uptake. The rates of efflux are reported as mean ± S.E.M. from at least 4 separate determinations conducted in duplicate.

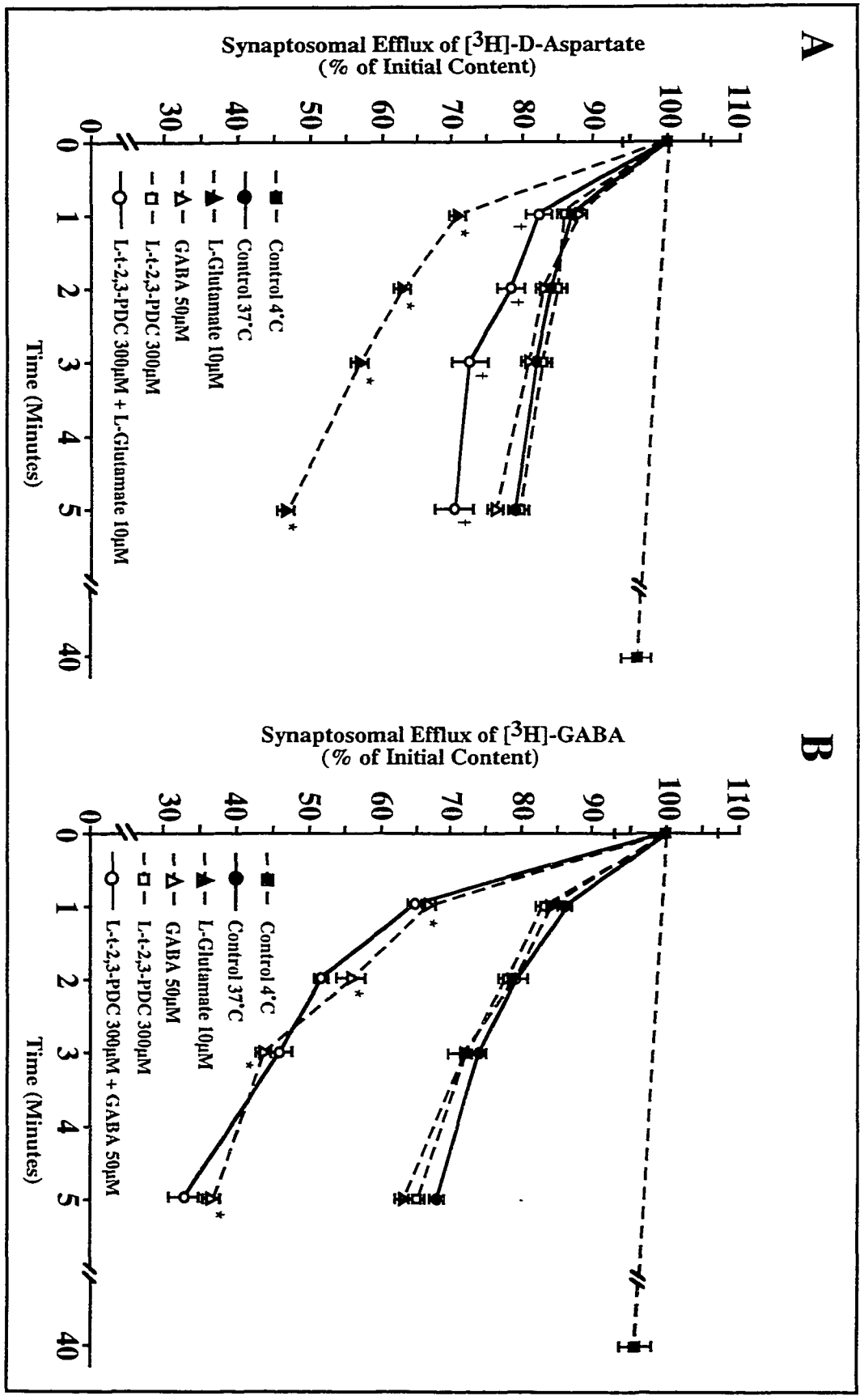


Figure 4.1: Time Courses for Synaptosomal Efflux of [3H]-Substrate

Figure 4.1: The time course for the efflux of either **A)** [^{3}H]-D-aspartate or **B)** [^{3}H]-GABA is reported as a percentage of the initial synaptosomal content (1552±65 pmol [^{3}H]-D-aspartate / mg protein or 3887±263 pmol [^{3}H]-GABA / mg protein). While the efflux of [^{3}H]-substrate was not detected from synaptosomes maintained at 4°C, the inclusion of L-glutamate or GABA at 37°C caused an efflux of over 50% of synaptosomal [^{3}H]-D-aspartate or [^{3}H]-GABA, respectively. In contrast, GABA did not stimulate the efflux of [^{3}H]-D-aspartate nor did L-glutamate potentiate the release of [^{3}H]-GABA. Interestingly, L-*trans*-2,3-PDC did not participate in heteroexchange on its own but it did significantly attenuate the efflux of [^{3}H]-D-aspartate caused by the inclusion of L-glutamate. L-*trans*-2,3-PDC had no effect on the efflux of [^{3}H]-GABA. Values are reported as mean ± S.E.M. from at least 4 determinations conducted in duplicate. Statistical comparisons were performed using the Alternate Welch t-test (GraphPad software, San Diego, CA): * $p<.0001$ versus Control efflux; † $p<.01$ vs L-Glutamate-induced efflux.

Figure 4.2: Transporter-Specific Efflux of [^{3}H]-Substrate from Synaptosomes

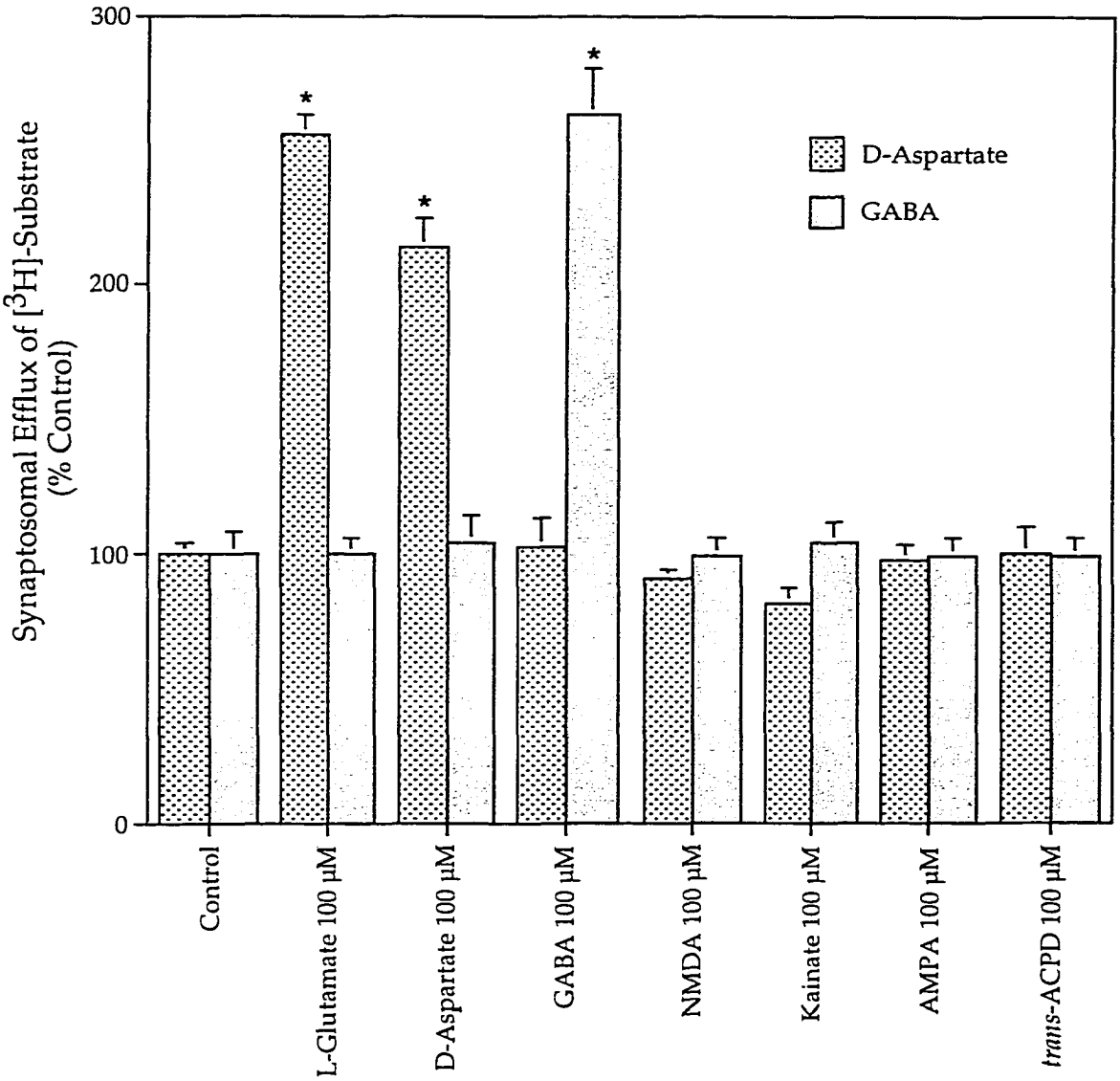


Figure 4.2: Efflux of radiolabeled substrate from synaptosomes containing either [^{3}H]-D-aspartate or [^{3}H]-GABA was determined as described in Chapter 2 and is expressed as percent of Control (i.e., efflux over a 2 min interval at 37°C in the absence of uptake inhibitors). Initial content of synaptosomes was 1552 ± 62 pmol [^{3}H]-D-aspartate / mg protein or 3887 ± 263 pmol [^{3}H]-GABA / mg protein. Control efflux rates were 114 ± 4 pmol / min / mg protein for [^{3}H]-D-aspartate and 354 ± 30 pmol / min / mg for [^{3}H]-GABA. Values are reported as mean ± S.E.M., n = 4-57 duplicate determinations. Statistical comparisons were made using an Alternate Welch t-test (GraphPad software, San Diego, CA); * $p<0.0005$ versus Control efflux.

Figure 4.3: Electrophysiological Recordings of Analogues in *Xenopus* Oocytes Expressing EAAT2.

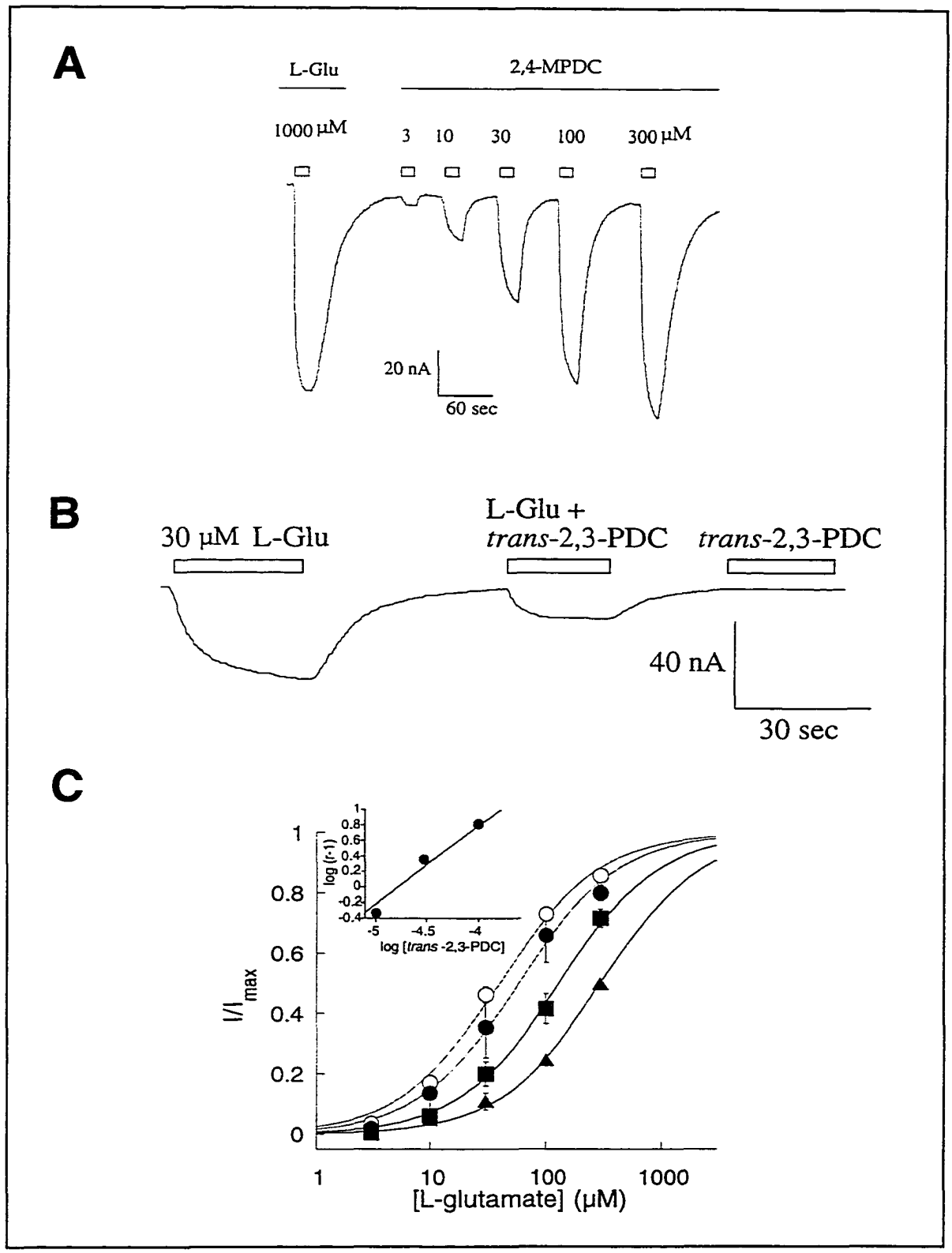


Figure 4.3: Actions of transported and nontransported analogues at EAAT2 were determined as described in Chapter 2. **A.** Currents induced by application of glutamate or 2,4-MPDC (duration indicated by bar above trace) in a representative *Xenopus* oocyte expressing EAAT2. 2,4-MPDC induced a dose-dependent and saturable current with a K_m of 45μM (membrane potential = -25 mV). **B.** Co-application of L-*trans*-2,3-PDC (30 μM) reduced glutamate-induced current without inducing current on its own. **C.** Schild analysis of the effect of L-*trans*-2,3-PDC on glutamate currents revealed competitive antagonism, K_d = 16 μM. Open circle - control; closed circle - 10 μM L-*trans*-2,3-PDC; closed square - 30 μM L-*trans*-2,3-PDC; closed triangle - 100 μM L-*trans*-2,3-PDC (conducted in collaboration with M.P. Kavanaugh, Vollum Institute).

Figure 4.4: Correlations Between Synaptosomal Efflux Rates and Currents Measured in *Xenopus* Oocytes Expressing EAAT1, EAAT2 and EAAT3.

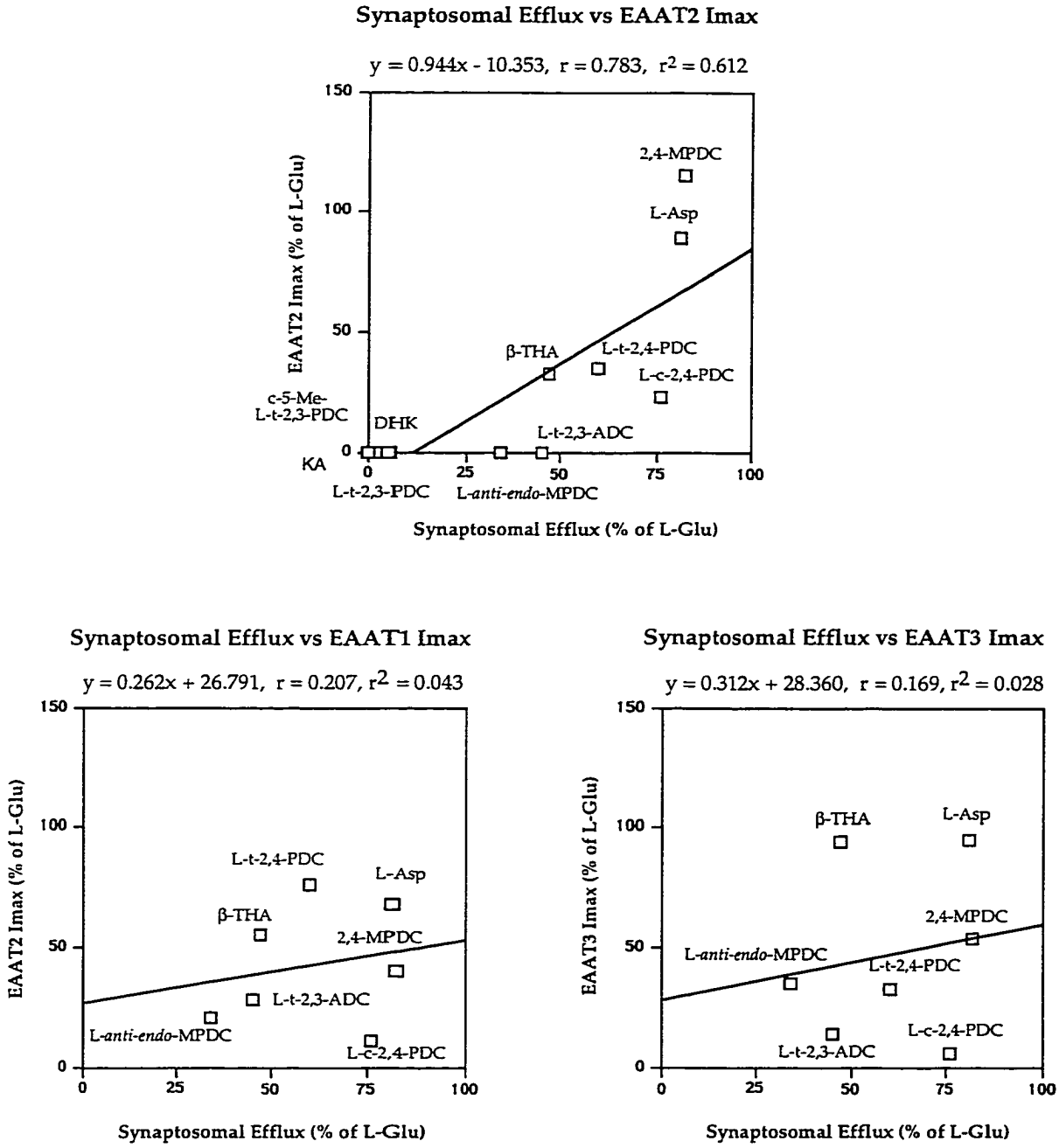


Figure 4.4: Correlations between inhibitor-induced synaptosomal efflux rates and inhibitor-mediated currents in oocytes were made using Cricket Graph III software (Islandia, NY). Efflux rates from synaptosomes were quantified as described in Chapter 2 and are reported as a percentage of the activity of L-glutamate at 37°C. Currents in voltage clamped *Xenopus* oocytes expressing EAAT1, EAAT2, and EAAT3 were measured as described in Chapter 2 and are reported as a percentage of the current (I_{max}) induced by L-glutamate. Graphs comparing synaptosomal efflux with currents observed at EAAT1 and EAAT3 do not include dihydrokainate, kainate, L-*trans*-2,3-PDC or *cis*-5-Me-L-*trans*-2,3-PDC since there was no activity detected for these inhibitors at either of these transporter clones. The greatest correlation between synaptosomal efflux rates and I_{max} currents measured in the cloned transporters was observed with EAAT2 which yielded an r^2 value of 0.612, compared with $r^2 = 0.043$ and $r^2 = 0.028$ for EAAT1 and EAAT3, respectively.

Table 4.2: Comparison of Synaptosomal and EAAT Pharmacology

	SYNAPTOSOMES		EAAT 1		EAAT 2		EAAT 3	
COMPOUND	K_i (μM)	efflux (% of Glu)	K_m (μM)	I_{max} (% of Glu)	K_m or K_i* (μM)	I_{max} (% of Glu)	K_m (μM)	I_{max} (% of Glu)
L-Glutamate	4.9 ± 1	100	20 ± 3	100	18 ± 3	100	28 ± 6	100
Dihydrokainate	28 ± 2	3.0 ± 1.5	n.d.	n.d.	9*	0	n.d.	n.d.
L-*trans*-2,3-PDC	33 ± 6	5.0 ± 1.5	n.d.	n.d.	12*	0	n.d.	n.d.
2,4-MPDC	6.8 ± 3	82 ± 9	87 ± 7	40 ± 4	45 ± 3	115 ± 3	85 ± 10	54 ± 2

Table 4.2: Analogues were evaluated as inhibitors and substrates in oocytes expressing EAAT1, EAAT2, and EAAT3 as described in Chapter 2. In the instances where compounds exhibited no substrate activity (*), K_i values were calculated by Schild analysis. In the heteroexchange studies, analogues were tested at concentrations ≈ 10 X greater than the K_i values for inhibition of D-aspartate uptake as described in Chapter 2. Values indicating substrate activity are reported as a percentage of the activity of L-glutamate at 37°C. The abbreviation n.d. denotes that no activity was detected.

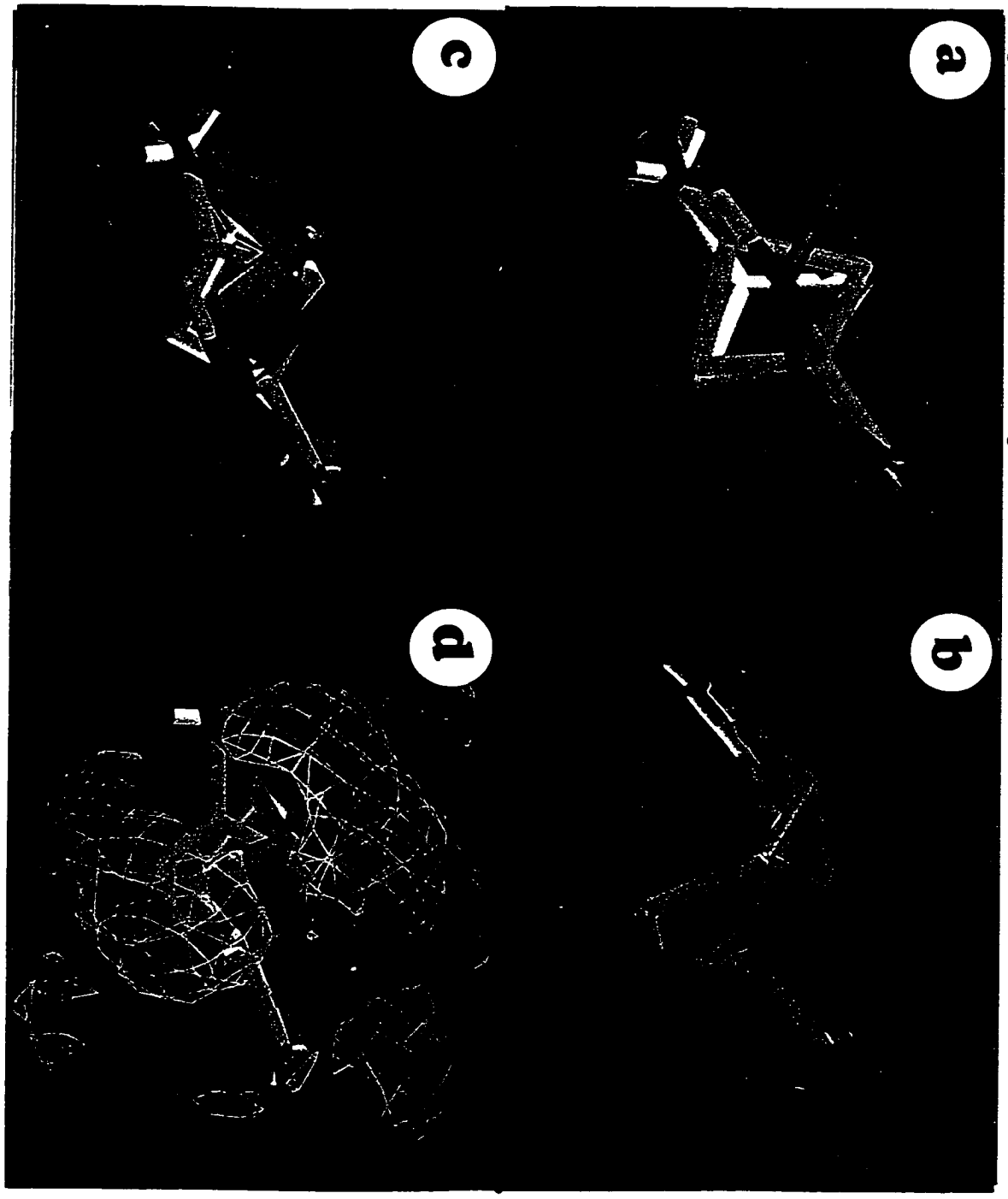


Figure 4.5: Molecular Modeling of Substrates and Nonsubstrates

Figure 4.5: Molecular modeling overlays using a three point fit of the amino group (purple), α-carboxylate carbon and distal carboxylate carbon (red oxygens) of **a)** substrates: L-glutamate (green), L-*trans*-2,4-PDC (red), *cis*-ACBD (white), and 2,4-MPDC (yellow); **b)** L-glutamate and nonsubstrates: L-glutamate (green), L-*anti-endo*-3,4-MPDC (blue), and L-*trans*-2,3-PDC (orange); **c)** composite of the substrates and nonsubstrates depicted in a) and b); **d)** the excess molecular volume of nonsubstrates *vs.* the molecular volume of substrates as depicted by the yellow grid. This region represents the excess steric bulk associated with the nontransportable inhibitors that is believed to interact with the transport protein in such a manner as to reduce the transportability of these compounds. All molecules are minimized conformations (using SYBYL molecular modeling software by Tripos). Hydrogens are omitted for clarity.

Chapter 5: Nonsubstrate Inhibitors Attenuate the Reversal of Glutamate Transporters in Synaptosomes Following a Metabolic Insult.

Introduction

High-affinity, sodium-dependent uptake systems are believed to play a central role in maintaining glutamate concentrations appropriate for normal synaptic transmission and below those that are excitotoxic. Ironically, under some pathological conditions these same transport systems may actually participate in the excitotoxic process by acting as sites of efflux of L-glutamate from intracellular compartments (Takahashi et al., 1997). Numerous studies have suggested that metabolic insults which compromise cellular energy levels can lead to the reversed action of the transporter and the movement of L-glutamate down its concentration gradient into the extracellular space (Kauppinen et al., 1988; Sanchez-Prieto and Gonzalez, 1988; Gemba et al., 1994; Szatkowski and Attwell, 1994; Longuemare and Swanson, 1995). This pathway, possibly in combination with alternative routes of L-glutamate efflux (Kimelberg et al., 1990), could contribute to the rise in extracellular glutamate levels observed in models of anoxia and ischemia (Benveniste et al., 1984; Roettiger and Lipton, 1996).

In this chapter we examined the efflux of [^{3}H]-D-aspartate, a selective substrate of the high-affinity, sodium-dependent glutamate carriers, from

synaptosomes in response to a metabolic insult (*i.e.*, potassium cyanide and iodoacetate). We demonstrated that the metabolic insult-mediated efflux of [^{3}H]-D-aspartate from the synaptosomes exhibited a timecourse similar to the heteroexchange-mediated efflux that was observed in Chapter 4. In addition, like the efflux of radioligand that was induced by L-glutamate, the metabolic inhibitor-induced efflux could be attenuated with nontransportable uptake inhibitors, e.g. L-trans-2,3-PDC and dihydrokainate. These results thus add further strength to the model of injury-induced efflux through the glutamate carriers and identify a novel strategy with which to attenuate this process.

Results and Discussion

Similar to what was observed when synaptosomes containing [^{3}H]-D-aspartate were exposed to 10 μM L-glutamate (Chapter 4), a stimulation of the efflux of pre-accumulated amino acid was also observed when synaptosomes were exposed to a chemical insult consisting of potassium cyanide (KCN, 5 mM) and iodoacetate (IOA, 1 mM) (Figure 5.1). The combination of an electron transport chain blocker (KCN) and a glycolytic inhibitor (IOA) has been employed in numerous studies as a metabolic insult and as a simplified chemical model of ischemia/anoxia because of its ability to inhibit respiration, deplete ATP levels, and erode membrane potentials (Kauppinen et al., 1988; Reiner et al., 1990; Zeevalk and Nicklas,

1991; Longuemare and Swanson, 1995). As illustrated in Figure 5.1A, the efflux of [^{3}H]-D-aspartate produced by KCN and IOA exhibited a time course very similar to that of glutamate-mediated heteroexchange. Over a 5 min period the synaptosomal content of [^{3}H]-D-aspartate was reduced to about 50% of its original level. Interestingly, an analogous increase in efflux was not observed when synaptosomes containing [^{3}H]-GABA were exposed to these same metabolic inhibitors (Figure 5.1B). Whether this lack of response was attributable to: *i*) the metabolism or sequestration of GABA in synaptic vesicles (unlikely, given the extent of the homoexchange-mediated efflux), *ii*) the GABA pool being less sensitive to metabolic insult, as suggested by (Hauptman et al., 1984), or *iii*) as a consequence of the inhibitor-induced efflux of [^{3}H]-GABA being masked by a larger basal rate of efflux (Figure 5.1B), remains to be determined. While the potential action of KCN and IOA at alternative sites can not be excluded, these results are consistent with previous demonstrations that the chemical inhibition of cellular respiration increases extracellular levels of L-glutamate in a variety of physiological preparations (Kauppinen et al., 1988; Sanchez-Prieto and Gonzalez, 1988; Zeevalk and Nicklas, 1991; Madl and Burgesser, 1993; Gemba et al., 1994).

In addition to an ability to block heteroexchange, the nontransportable inhibitors L-*trans*-2,3-PDC and dihydrokainate also attenuated the efflux of [^{3}H]-D-aspartate that was induced by the metabolic inhibitors. This finding suggests that the same transport system is involved in both the processes

of heteroexchange and transporter reversal during metabolic insult (Table 5.1, Figure 5.1A). In contrast to the ability of an analogue to block glutamate-mediated heteroexchange by directly competing with extracellular L-glutamate for the carrier binding sites, the results lead to the conclusion that the occupation of external binding sites by a nontranslocatable analogue reduces the rate at which an internal substrate can egress through this system. Mechanistically, this process is also distinct from the reduction in the metabolic inhibitor-induced efflux of [^{3}H]-D-aspartate from cultured astrocytes that was produced intracellularly by pre-equilibrating the cells with competitive inhibitors that were also substrates (D,L-β-THA and L-*trans*-2,4-PDC) (Longuemare and Swanson, 1995). It therefore appears that the binding of a nontransportable analogue may essentially trap the carrier binding site on the extracellular face of the plasma membrane. In support of this interpretation, a reduction in the metabolic inhibitor-induced efflux was observed only with the nontransportable blockers and not with substrate inhibitors (*e.g.*, L-*anti-endo*-3,4-MPDC, 2,4-MPDC, D,L-β-THA and L-*trans*-2,4-PDC) (Table 5.1). In fact, when 2,4-MPDC was included in the assay with KCN and IOA, the resulting efflux of amino acid was significantly higher than that produced by KCN and IOA alone. Thus, potent substrates can even exacerbate the transporter reversal caused by a metabolic insult. It is also acknowledged that the attenuation of the [^{3}H]-D-aspartate efflux by L-*trans*-2,3-PDC was not complete (e.g., about 50% at 5 min, Figure 5.1A), suggesting that a

portion of the insult-induced efflux may also have been occurring via either an EAAT subtype insensitive to L-*trans*-2,3-PDC (see below) or through a non-EAAT mediated mechanism (Kimelberg et al., 1990).

Interestingly, both dihydrokainate (Pines et al., 1992; Arriza et al., 1994; Vandenberg, 1998) and L-*trans*-2,3-PDC (see Chapter 4) appear to be selective inhibitors of the EAAT2/GLT-1 transporter subtype believed to be localized primarily on glia in the CNS (for review see: Gegelashvili and Schousboe, 1998). The specificity of these analogues would, in turn, suggest that at least some portion of the observed efflux of [^{3}H]-D-aspartate originated from glial elements within the synaptosomal preparation (see: Chapter 4, Henn et al., 1976; Nakamura et al., 1993). It is also worth noting that the inclusion of L-*anti-endo*-3,4-MPDC, a potent nontransportable inhibitor of EAAT2 and substrate of EAAT1 and EAAT3 (Chapter 4), did not attenuate the metabolic insult-induced efflux. The potential for this analogue to inhibit the observed efflux at EAAT2 may have been counteracted by its ability to act as a substrate, and thus participate in heteroexchange, at EAAT1 and EAAT3. The presence of glial transporters in this subcellular fraction is also consistent with the demonstration that synaptosomes prepared from mice deficient in GLT-1 exhibited a marked loss in the ability to transport L-glutamate (Tanaka et al., 1997), as was discussed in Chapter 4. While more selective inhibitors and additional kinetic studies will be needed to quantitatively delineate the specific cellular pools and individual proteins that contribute to excitatory amino

acid efflux during metabolic insult, these findings highlight the use of nontransportable uptake inhibitors as a novel strategy to regulate this process.

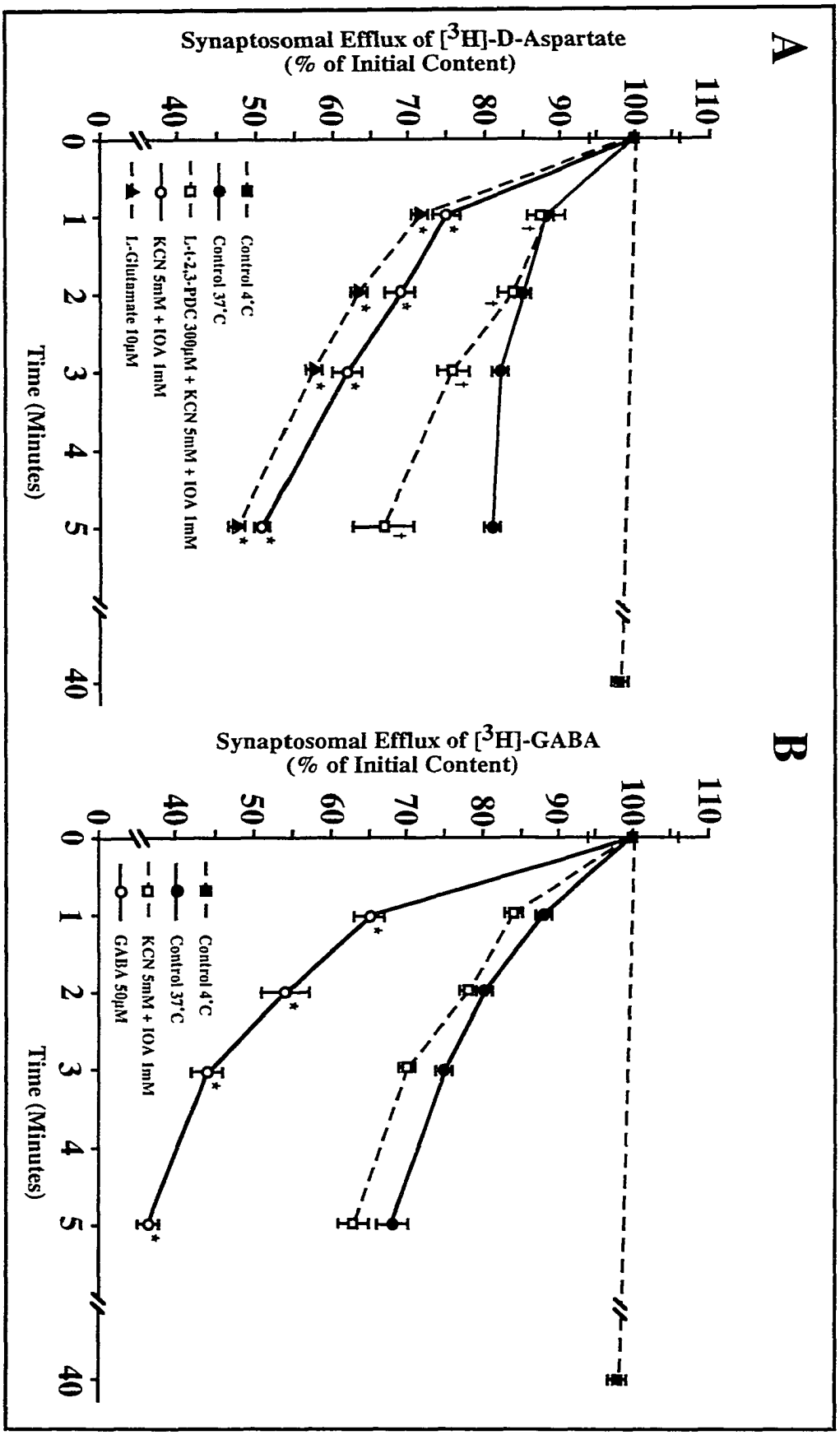


Figure 5.1: Time Courses for Synaptosomal Efflux of [^{3}H]-Substrate

Figure 5.1: Synaptosomal efflux of **A)** [^{3}H]-D-aspartate, or **B)** [^{3}H]-GABA, reported as a percentage of initial radiolabel content (1552±65 pmol [^{3}H]-D-aspartate/mg protein and 3887±263 pmol [^{3}H]-GABA/mg protein), was followed over a period of 5 min in the presence and absence of the indicated compounds. Synaptosomes maintained at 4°C did not exhibit a significant loss of [^{3}H]-substrate, while the inclusion of L-glutamate or GABA at 37°C caused an efflux of over 50% of synaptosomal [^{3}H]-D-aspartate or [^{3}H]-GABA, respectively. A metabolic insult of 5 mM KCN and 1mM IOA (iodoacetate) caused an efflux of [^{3}H]-D-aspartate similar in extent to that caused by 10 μM L-glutamate. In contrast, 5 mM KCN and 1mM IOA caused only a slight, albeit statistically nonsignificant, increase in the efflux of [^{3}H]-GABA. Interestingly, inclusion of the non-transportable uptake inhibitor L-*trans*-2,3-PDC significantly attenuated the efflux of [^{3}H]-D-aspartate that was caused by the metabolic insult. Values are reported as mean ± S.E.M. from at least 4 duplicate determinations. Statistical comparisons were made using an Alternate Welch t-test (GraphPad, San Diego, CA) that does not assume equal SD's: * $p<.0001$ versus Control efflux; † $p<.005$ versus KCN- and IOA-induced efflux.

Table 5.1: Attenuation of KCN- and IOA-Induced Efflux of [^{3}H]-D-Aspartate from Synaptosomes by Nontransportable Inhibitors.

Compounds	[^{3}H]-D-Aspartate Efflux (% of Control)
Control	100 ± 4
10 μM L-Glutamate	233 ± 10 [a]
5 mM KCN & 1 mM IOA	224 ± 18 [a]
5 mM KCN & 1 mM IOA & 300 μM L-*trans*-2,3-PDC	117 ± 12 [b]
5 mM KCN & 1 mM IOA & 300 μM DHK	121 ± 17 [b]
5 mM KCN & 1 mM IOA & 50 μM L-*anti-endo*-3,4-MPDC	220 ± 18 [a]
5 mM KCN & 1 mM IOA & 75 μM 2,4-MPDC	305 ± 30 [a]
5 mM KCN & 1 mM IOA & 20 μM D,L-β-THA	280 ± 42 [a]
5 mM KCN & 1 mM IOA & 15 μM L-*trans*-2,4-PDC	284 ± 32 [a]

Table 5.1: Efflux of synaptosomal [^{3}H]-D-aspartate was quantified over a 2 min period at 37°C and is reported as percent of Control (*i.e.*, efflux in the absence of added substrates). Initial content of the synaptosomes was 1552±62 pmol [^{3}H]-D-aspartate, and Control rate of efflux was 114±4 pmol/min/mg protein [^{3}H]-D-aspartate. The efflux of [^{3}H]-D-aspartate induced by a metabolic insult of 5 mM KCN and 1 mM IOA was significantly attenuated by the nontransportable uptake inhibitors L-*trans*-2,3-PDC and dihydrokainate, but not by the transportable inhibitors L-*anti-endo*-3,4-MPDC, 2,4-MPDC, D,L-β-THA and L-*trans*-2,4-PDC. Values are reported as mean ± S.E.M., n= 4-57 duplicate determinations. Statistical comparisons were made using an Alternate Welch t-test (GraphPad, San Diego, CA): a, $p<0.0001$ versus Control efflux. b, $p<0.0005$ versus KCN and IOA induced efflux.

Chapter 6: Summary and Conclusions

Thirty years ago, few would have accepted the α-amino acid L-glutamate as a neurotransmitter, and much less as the primary excitatory transmitter at all synaptic sites within the mammalian central nervous system. Since this time, selective ligands have been used to delineate individual subtypes of receptors, glutamatergic pathways have been mapped in the brain, and a separate transport system was identified that terminates the actions of the transmitter in the synaptic cleft. With the explosion in the field of molecular biology within the last ten years, the study of the pharmacology, physiology and structural analysis of the glutamate receptors and transporters has been taken to new heights. Well over a dozen individual subunits have been cloned for the three families of ionotropic receptors, and at least six subtypes of metabotropic receptors have been identified (for review see: Cotman et al., 1995; Dingledine et al., 1999). Pharmacological developments have not been far behind with several hundred agonists and antagonists developed, many with selectivity for individual subtypes of EAA receptors. The year 1992 brought the initial cloning of three high-affinity glutamate transporters, GLAST, GLT-1, and EAAC1 from animal tissue (Kanai and Hediger, 1992; Pines et al., 1992; Storck et al., 1992), and the isolation of three counterparts from human tissue, EAAT1, EAAT2, and EAAT3, respectively, followed

shortly thereafter (Arriza et al., 1994). To date, five human subtypes have been identified, most recently EAAT4 and EAAT5 (Fairman et al., 1995; Arriza et al., 1997). The pharmacology of these transporters, however, has remained in a relative state of infancy as compared with that of the EAA receptors.

Bearing this in mind, the first portion of this work was directed towards the identification of potent inhibitors of the high-affinity glutamate transporters present in forebrain synaptosomes. It was previously demonstrated that the incorporation of L-glutamate or L-aspartate into a constrained ring system could produce potent and selective inhibitors (Bridges et al., 1991; Bridges et al., 1993). This strategy was used in the present study to identify three novel types of inhibitors based on the pyrrolidine ring structure: 2,3-pyrrolidine dicarboxylates, 3,4-methanopyrrolidine dicarboxylates and 2,4-methanopyrrolidine dicarboxylate (Bridges et al., 1994; Willis et al., 1996; Esslinger et al., 1998).

- **L-*trans*-2,3-PDC was found to be a moderately potent, competitive inhibitor of high-affinity, synaptosomal uptake of L-glutamate.** Of the series of 2,3-pyrrolidine dicarboxylates that was prepared and tested, L-*trans*-2,3-PDC was found to be the only isomer with activity for inhibiting the uptake of [^{3}H]-D-aspartate into synaptosomes. This constrained analogue of L-aspartate acted with a similar potency as the well known uptake inhibitor dihydrokainate.

- **L-*anti-endo*-3,4-MPDC was demonstrated to be a potent, competitive inhibitor of the synaptosomal glutamate uptake system.** Of the three bicyclic diastereomers of L-3,4-methanopyrrolidine dicarboxylate that were evaluated for the ability to inhibit the high-affinity synaptosomal glutamate transporter, only L-*anti-endo*-3,4-MPDC was found to show activity. This isomer relied on the addition of a 3,4-methano bridge to the pyrrolidine ring in order to mimic the folded conformation of L-*trans*-2,4-PDC. L-*anti-endo*-3,4-MPDC inhibited the uptake of [^{3}H]-D-aspartate to a similar extent as the endogenous substrate L-glutamate.

- **2,4-methanopyrrolidine dicarboxylate (2,4-MPDC) was found to be a potent, competitive inhibitor of the synaptosomal transport of L-glutamate.** By adding further conformational constraint to the pyrrolidine ring via the incorporation of a methano bridge, this bicyclic analogue of L-glutamate was designed to mimic the extended conformation of L-*trans*-2,4-PDC. 2,4-MPDC inhibited synaptosomal glutamate transport with similar potency as L-glutamate and L-*anti-endo*-3,4-MPDC.

- **Molecular modeling studies were performed to compare the proposed binding conformations of L-*trans*-2,3-PDC, L-*anti-endo*-3,4-MPDC and 2,4-MPDC with those of L-glutamate and L-*trans*-2,4-PDC.** Using a three point

fit of the amino group, α-carboxylate carbon and distal carboxylate carbon, a high degree of overlap was found for the functional group positionings of the five mentioned inhibitors. The results suggest that while the arrangements of the functional groups are important for transporter binding, important considerations can also be made regarding the stereoselectivity of the binding site and the tolerance for additional steric volume occupied by the backbones of the constrained inhibitors.

While numerous studies in the past have used analogues of L-glutamate to probe the functions of the EAA transporters (for review see: Bridges et al., 1999), very few have attempted to distinguish these analogues on the basis of substrates and nonsubstrates. In other words, it was presumed that inhibitors of glutamate uptake could be differentiated between molecules that compete with L-glutamate for the binding site and are then transported themselves (substrate inhibitors), and inhibitors that compete for binding but are not translocated (nontransportable inhibitors). By utilizing the process of heteroexchange, a method was developed for rapidly delineating substrates from nonsubstrate inhibitors of the synaptosomal transporters. Thus, following the initial identification of the inhibitory properties of a series of analogues, additional investigations into the mechanism of substrate specificity of glutamate transport were

conducted using these molecules (Chamberlin et al., 1998; Esslinger et al., 1998; Koch et al., 1999).

- **The process of transporter-mediated heteroexchange was exploited in synaptosomes in order to rapidly distinguish transportable from nontransportable inhibitors.** It was demonstrated that the endogenous substrate L-glutamate could cause the efflux of radiolabeled D-aspartate that had been previously sequestered into the synaptosomes. The observed efflux appears to represent the transporter mediated-exchange of internal for external substrate. These findings were supported by the observations that the transporter-specific substrates L-glutamate and L-aspartate could stimulate exchange while GABA, NMDA, kainate and AMPA could not.

- **A wide range of substrate activities was found within the library of conformationally constrained inhibitors.** A number of potent inhibitors were identified that shared similar positionings of their functional groups yet exhibited markedly different capacities to serve as substrates for the high-affinity glutamate transport system. In addition, the substrate activities did not necessarily correlate with the ability of the compounds to bind to the transporter. It was demonstrated that 2,4-MPDC, L-*anti-endo*-3,4-MPDC, L-*trans*-2,4-PDC, *cis*-ACBD and D,L-β-THA act as substrates for

the synaptosomal glutamate uptake system. In contrast, L-*trans*-2,3-PDC, *cis*-5-Me-L-*trans*-2,3-PDC and dihydrokainate proved to be competitive inhibitors of uptake that did not exhibit activity as substrates.

- **Molecular modeling comparisons were used to refine the pharmacophore model of the substrate binding site on the transporter and to explore the chemical properties that define the substrate activities of inhibitors.** It was found within the library of analogues that conformational restriction does not necessarily dictate substrate activity. Regions of excess steric volume associated with the carbon backbones of the nonsubstrates were identified that were distinct relative to the space occupied by the backbones of the substrates. It is likely that steric excess in these specific positions represents a determining factor as to whether or not the analogue can be transported. While similar positionings of the functional groups may allow binding to occur, it can be concluded that the excess volume occupied by the backbones of the nontransportable analogues may interact in an unfavorable manner with specific domains on the transporter protein that govern translocation.

- **The pharmacological profile of the synaptosomal preparation exhibited the greatest correspondence with EAAT2, a transporter subtype believed to be primarily of glial origin.** Electrophysiological characterization of

transport into *Xenopus* oocytes expressing the human transporter clones provided an alternative approach with which to corroborate the conclusions derived from the heteroexchange studies. When the same library of analogues was characterized electrophysiologically at EAAT1, EAAT2, and EAAT3, it was found that the pharmacological profile of substrate and nonsubstrate inhibitors of the synaptosomal system exhibited the greatest similarity with the EAAT2 subtype. This conclusion is quite interesting as EAAT2 is thought to be expressed primarily in astrocytes.

Numerous studies have demonstrated that metabolic insults, such as anoxia and ischemia, which compromise cellular energy levels can lead to the reversed action of the high-affinity glutamate transporters and the movement of L-glutamate down its concentration gradient into the extracellular space (Kauppinen et al., 1988; Sanchez-Prieto and Gonzalez, 1988; Gemba et al., 1994; Szatkowski and Attwell, 1994; Longuemare and Swanson, 1995). Elevated levels of extracellular L-glutamate can in turn result in the overactivation of EAA receptors and thus induce excitotoxic neuronal injury (Choi, 1994; Rothman and Olney, 1995). In the final chapter of the study, it was demonstrated that a metabolic insult (potassium cyanide and iodoacetate) designed to mimic conditions during ischemia and anoxia, could be used to induce the efflux of amino acid via the high-affinity glutamate transporters. In addition, selective uptake

inhibitors were used in an attempt to attenuate the transporter reversal (Koch et al., 1999).

• **A chemically-induced metabolic insult causes the reversal of high-affinity glutamate transporters in synaptosomes.** Synaptosomes containing radiolabeled D-aspartate were exposed to a metabolic insult consisting of an electron transport chain blocker (potassium cyanide) and a glycolytic inhibitor (iodoacetate). The resulting efflux of amino acid was similar in extent to that which was observed during glutamate-mediated heteroexchange.

• **Nontransportable inhibitors can be used to attenuate the metabolic inhibitor-induced efflux of amino acid from synaptosomes.** The substrate inhibitors L-*anti-endo*-3,4-MPDC, 2,4-MPDC, D,L-β-THA and L-*trans*-2,4-PDC had either no effect or in fact exacerbated the metabolic inhibitor-induced efflux, as was the case for 2,4-MPDC. On the other hand, the nontransportable inhibitors L-*trans*-2,3-PDC and dihydrokainate, which share a similar ability to block heteroexchange, attenuated the efflux of D-aspartate observed during the metabolic insult. These results lead to the conclusion that the occupation of extracellular transporter binding sites by nonsubstrate analogues reduces the rate at which an intracellular substrate can egress through this system. In addition, these findings add further

strength to the model of glutamate transporter reversal during a metabolic insult and identify a novel strategy with which to attenuate this process.

The work detailed in this study will no doubt contribute to the further characterization and understanding of the important physiological roles of the high-affinity glutamate transporters in the mammalian central nervous system. From a medical science perspective, progress in this direction is critical in lieu of the vast number of pathologies in which L-glutamate appears to be involved. The need is apparent, however, for a greater number of more highly selective inhibitors of the glutamate transporters. More detailed molecular modeling studies as well as further modifications to the pyrrolidine dicarboxylate theme will undoubtedly provide an even greater array of analogues with which to probe the pharmacophores of the individual transporter subtypes. Selective substrates and nontransportable inhibitors of the EAATs will be particularly useful in elucidating the contribution of the individual subtypes to normal signaling processes as well as their involvement in pathological scenarios. The development of more potent and selective nontransportable inhibitors may also provide a novel therapeutic avenue for the treatment of conditions such as stroke and trauma in which transporter reversal and excitotoxic levels of L-glutamate may be a concern.

Bibliography

Arriza, J. L., Eliasof S., Kavanaugh M. P. and Amara S. G. (1997). Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. *Proc. Natl. Acad. Sci. USA* **94**: 4155-4160.

Arriza, J. L., Fairman W. A., Wadiche J. I., Murdoch G. H., Kavanaugh M. P. and Amara S. G. (1994). Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. *J. Neurosci.* **14**(9): 5559-5569.

Arunlakshana, O. and Schild H. O. (1959). Some qualitative uses of drug antagonists. *Br. J. Pharmacol.* **14**: 48-58.

Arzberger, T., Krampfl K., Leimgruber S. and Weindl A. (1997). Changes of NMDA receptor subunit (NR1, NR2B) and glutamate transporter (GLT1) mRNA expression in huntington's disease-an in situ hybridization study. *J. Neuropath. Expt. Neurol.* **56**(4): 440-454.

Balcar, V. J. and Johnston G. A. (1972). The structural specificity of the high affinity uptake of L-glutamate and L-aspartate by rat brain slices. *J. Neurochem.* **19**(11): 2657-2666.

Benveniste, H., Drejer J., Schousboe A. and Diemer N. H. (1984). Elevation of the extracellular concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. *J. Neurochem.* **43**: 1369-1374.

Booth, R. F. G. and Clark J. B. (1978). A rapid method for the preparation of relatively pure metabolically competent synaptosomes from rat brain. *Biochem. J.* **176**: 365-370.

Bridges, R. J., Kavanaugh M. P. and Chamberlin A. R. (1999). A pharmacological review of competitive inhibitors and substrates of high-affinity, sodium-dependent glutamate transport in the central nervous system. *Curr. Pharm. Des.* **5**: 363-379.

Bridges, R. J., Lovering F. E., Humphrey J. M., Stanley M. S., Blakely T. N., Christofaro M. F. and Chamberlin A. R. (1993). Conformationally restricted inhibitors of the high affinity L-glutamate transporter. *Bioorg. Med. Chem. Lett.* **3**(1): 115-121.

Bridges, R. J., Lovering F. E., Koch H., Cotman C. W. and Chamberlin A. R. (1994). A conformationally constrained competitive inhibitor of the sodium-dependent glutamate transporter in forebrain synaptosomes: L-anti-endo-3,4-methanopyrrolidine dicarboxylate. *Neurosci. Lett.* **174**: 193-197.

Bridges, R. J., Stanley M. S., Anderson M. W., Cotman C. W. and Chamberlin A. R. (1991). Conformationally defined neurotransmitter analogues. Selective inhibition of glutamate uptake by one pyrrolidine-2,4-dicarboxylate diastereomer. *J. Med. Chem.* **34**: 717-725.

Chamberlin, A. R., Koch H. P. and Bridges R. J. (1998). Design and synthesis of conformationally constrained inhibitors of high-affinity, sodium-dependent glutamate transporters. *Methods Enzymol.* **296**: 175-189.

Choi, D. W. (1988). Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**: 623-634.

Choi, D. W. (1994). Glutamate receptors and the induction of excitotoxic neuronal death. *Prog. Brain Res.* **100**: 47-51.

Choi, D. W. (1995). Calcium: still center-stage in hypoxic-ischemic neuronal death. *TINS* **18**: 58-60.

Christensen, H. N. (1975). Biological Transport. Massachusetts, Reading.

Conn, P. J. and Pin J. P. (1997). Pharmacology and functions of metabotropic glutamate receptors. *Annu. Rev. Pharmacol. Toxicol.* **37**: 205-237.

Cotman, C. W., Kahle J. S., Miller S. E., Ulas J. and Bridges R. J. (1995). Excitatory amino acid neurotransmission. Psychopharmacology: The Fourth Generation of Progress. New York, Raven Press. 75-85.

Curtis, D. R., Phillis J. W. and Watkins J. C. (1959). Chemical excitation of spinal neurons. *Nature* **183**: 611.

Dingledine, R., Borges K., Bowie D. and Traynelis S. F. (1999). The glutamate receptor ion channels. *Pharmacol. Rev.* **51**(1): 7-61.

Dowd, L. A., Coyle A. J., Rothstein J. D., Pritchett D. B. and Robinson M. B. (1996). Comparison of Na^+-dependent glutamate transport activity in synaptosomes, C6 glioma, and xenopus oocytes expressing excitatory amino acid carrier 1 (EAAC1). *Mol. Pharm.* **49**: 465-473.

Erecinska, M. and Troeger M. B. (1986). Amino acid neurotransmitters in the CNS. Characteristics of the acidic amino acid exchange. *FEBS Lett.* **199**(1): 95-99.

Esslinger, C. S., Koch H. P., Kavanaugh M. P., Philips D. P., Chamberlin A. R., Thompson C. M. and Bridges R. J. (1998). Structural determinants of substrates and inhibitors: probing glutamate transporters with 2,4-methanopyrrolidine-2,4-dicarboxylate. *Bioorg. Med. Chem. Lett.* **8**: 3101-3106.

Fairman, W. A., Vandenberg R. J., Arriza J. L., Kavanaugh M. P. and Amara S. G. (1995). An excitatory amino-acid transporter with properties of a ligand-gated chloride channel. *Nature* **375**: 599-603.

Fletcher, E. J., Mewett K. N., Drew C. A., Allan R. D. and Johnston G. A. R. (1991). Inhibition of high affinity L-glutamic acid uptake into rat cortical synaptosomes by the conformationally restricted analogue of glutamic acid, *cis*-1-aminocyclobutane-1,3-dicarboxylic acid. *Neurosci. Lett.* **121**: 133-135.

Garlin, A. B., Sinor A. D., Sinor J. D., Jee S. H., Grinspan J. B. and Robinson M. B. (1995). Pharmacology of sodium-dependent high-affinity L-^{3}H-glutamate transport in glial cultures. *J. Neurochem.* **64**: 2572-2580.

Gegelashvili, G. and Schousboe A. (1997). High affinity glutamate transporters: regulation of expression and activity. *Mol. Pharm.* **52**: 6-15.

Gegelashvili, G. and Schousboe A. (1998). Cellular distribution and kinetic properties of high-affinity glutamate transporters. *Brain Res. Bull.* **45**(3): 233-238.

Gemba, T., Oshima T. and Ninomiya M. (1994). Glutamate efflux via the reversal of the sodium-dependent glutamate transporter caused by glycolytic inhibition in rat cultured astrocytes. *Neuroscience* **63**(3): 789-795.

Griffiths, R., Dunlop J., Gorman A., Senior J. and Grieve A. (1994). L-trans-pyrrolidine-2,4-dicarboxylate and cis-1-aminocyclobutane-1,3-dicarboxylate behave as transportable, competitive inhibitors of the high-affinity glutamate transporters. *Biochem. Pharm.* **47**(2): 267-274.

Hauptman, M., Nelson D., Wilson D. F. and Erecinska M. (1984). Neurotransmitter Amino Acids in the CNS. II. Some Changes in Amino Acid Levels in Rat Brain Synaptosomes During and After In Vitro Anoxia and Simulated Ischemia. *Brain Res.* **304**: 23-35.

Henn, F. A., Anderson D. J. and Rustad D. G. (1976). Glial contamination of synaptosomal fractions. *Brain Res.* **101**: 341-344.

Humphrey, J. M., Bridges R. J., Hart J. A. and Chamberlin A. R. (1994). 2,3-Pyrrolidinedicarboxylates as neurotransmitter conformer mimics: enantioselective synthesis via chelation-controlled enolate alkylation. *J. Org. Chem.* **59**: 2467-2472.

Johnston, G. A. R., Kennedy S. M. E. and Twitchin B. (1979). Action of the neurotoxin kainic acid on high affinity uptake of L-glutamic acid in rat brain slices. *J. Neurochem.* **32**: 121-127.

Kanai, Y. and Hediger M. A. (1992). Primary structure and functional characterization of a high-affinity glutamate transporter. *Nature* **360**: 467-471.

Kauppinen, R. A., McMahon H. T. and Nicholls D. G. (1988). Ca2+ -dependent and Ca2+ -independent glutamate release, energy status and cytosolic free Ca2+ concentration in isolated nerve terminals following metabolic inhibition: possible relevance to hypoglycaemia and anoxia. *Neuroscience* **27**(1): 175-182.

Kimelberg, H. K., Goderie S. K., Pang S. and Waniewski R. A. (1990). Swelling-induced release of glutamate, aspartate, and taurine from astrocyte cultures. *J. Neurosci.* **10**(5): 1583-1591.

Koch, H. P., Chamberlin A. R. and Bridges R. J. (1999). Nontransportable inhibitors attenuate the reversal of glutamate uptake in synaptosomes following a metabolic insult. *Mol. Pharm.* 55(6): 1044-1048.

Koch, H. P., Kavanaugh M. P., Esslinger C. S., Zerangue N., Humphrey J. M., Amara S. G., Chamberlin A. R. and Bridges R. J. (1999). Differentiation of substrate and nonsubstrate inhibitors of the high-affinity, sodium-dependent glutamate transporters. *Mol. Pharm.* **(submitted).**

Kuhar, M. J. and Zarbin M. A. (1978). Synaptosomal transport: a chloride dependence for choline, GABA, glycine and several other compounds. *J. Neurochem.* **31**: 251-256.

Leigh, P. N. and Meldrum B. S. (1996). Excitotoxicity in ALS. *Neurol.* **47**(6 Suppl 4): S221-227.

Levy, L. M., Warr O. and Attwell D. (1998). Stoichiometry of the glial glutamate transporter GLT-1 expressed inducibly in a chinese hamster ovary cell line selected for low endogenous Na+-dependent glutamate uptake. *J. Neurosci.* **18**(23): 9620-9628.

Longuemare, M. C. and Swanson R. A. (1995). Excitatory amino acid release from astrocytes during energy failure by reversal of sodium-dependent uptake. *J. Neurosci. Res.* **40**: 379-386.

Madl, J. E. and Burgesser K. (1993). Adenosine triphosphate depletion reverses sodium-dependent, neuronal uptake of glutamate in rat hippocampal slices. *J. Neurosci.* **13**(10): 4429-4444.

Nakamura, Y., Iga K., Shibata T., Shudo M. and Kataoka K. (1993). Glial plasmalemmal vesicles: a subcellular fraction from rat hippocampal homogenate distinct from synaptosomes. *Glia* **9**: 48-56.

Nakamura, Y., Kataoka K., Ishida M. and Shinozaki H. (1993). (2S, 3S, 4R)-2-(carboxycyclopropyl)glycine, a potent and competitive inhibitor of both glial and neuronal uptake of glutamate. *Neuropharm.* **32**(9): 833-837.

Olney, J. W., Wozniak D. F. and Farber N. B. (1997). Excitotoxic degeneration in Alzheimer's disease: new hypothesis and new therapeutic strategies. *Arch. Neurol.* **54**(10): 1234-1240.

Pines, G., Danbolt N. C., Bjoras M., Zhang Y., Bendahan A., Eide L., Koepsell H., Storm-Mathisen J., Seeberg E. and Kanner B. I. (1992). Cloning and expression of a rat brain L-glutamate transporter. *Nature* **360**: 464-467.

Reiner, P. B., Laycock A. G. and Doll C. J. (1990). A pharmacological model of ischemia in the hippocampal slice. *Neurosci. Lett.* **119**: 175-178.

Robinson, M. B. (1998). Examination of glutamate transporter heterogeneity using synaptosomal preparations. *Methods Enzymol.* **296**: 189-202.

Roettiger, V. and Lipton P. (1996). Mechanism of glutamate release from rat hippocampal slices during in vitro ischemia. *Neuroscience* **75**(3): 677-685.

Rothman, S. M. and Olney J. W. (1987). Excitotoxicity and the NMDA receptor. *TINS* **10**(7): 299-302.

Rothman, S. M. and Olney J. W. (1995). Excitotoxicity and the NMDA receptor-still lethal after eight years. *TINS* **18**: 57-58.

Rudnick, G. (1998). Bioenergetics of neurotransmitter transport. *J. Bioenerg. Biomemb.* **30**(2): 173-185.

Sanchez-Prieto, J. and Gonzalez P. (1988). Occurrence of a large Ca2+ - independent release of glutamate during anoxia in isolated nerve terminals (synaptosomes). *J. Neurochem.* **50**: 1322-1324.

Shimamoto, K., LeBrun B., Yasuda-Kamatani Y., Sakaitani M., Shigeri Y., Yumoto N. and Nakajima T. (1998). D,L-threo-β-benzyloxyaspartate, a potent blocker of excitatory amino acid transporters. *Mol. Pharm.* **53**: 195-201.

Storck, T., Schulte S., Hofmann K. and Stoffel W. (1992). Structure, expression, and functional analysis of a Na+-dependent glutamate/aspartate transporter from rat brain. *Proc. Natl. Acad. Sci. USA* **89**: 10955-10959.

Szatkowski, M. and Attwell D. (1994). Triggering and execution of neuronal death in brain ischaemia: two phases of glutamate release by different mechanisms. *TINS* **17**(9): 359-365.

Takahashi, M., Billups B., Rossi D., Sarantis M., Hamann M. and Attwell D. (1997). The role of glutamate transporters in glutamate homeostasis in the brain. *J. Expt. Biol.* **200**: 401-409.

Tanaka, K., Watase K., Manabe T., Yamada K., Watanabe M., Takahashi K., Iwama H., Nishikawa T., Ichihara N., Kikuchi T., et al. (1997). Epilepsy and exacerbation of brain injury in mice lacking the glutamate transporter GLT-1. *Science* **276**: 1699-1702.

Vandenberg, R. J. (1998). Molecular pharmacology and physiology of glutamate transporters in the central nervous system. *Clin. Expt. Pharmacol. Physiol.* **25**: 393-400.

Vandenberg, R. J., Mitrovic A. D., Chebib M., Balcar V. J. and Johnston G. A. R. (1997). Contrasting modes of action of methylglutamate derivatives on the excitatory amino acid transporters, EAAT1 and EAAT2. *Mol. Pharm.* **51**: 809-815.

Volterra, A., Bezzi P., Rizzini B. L., Trotti D., Ullensvang K., Danbolt N. C. and Racagni G. (1996). The competitive transport inhibitor L-trans-pyrrolidine-2,4-dicarboxylate triggers excitotoxicity in rat cortical neuron-astrocyte co-cultures via glutamate release rather than uptake inhibition. *Eur. J. Neurosci.* **8**: 2019-2028.

Willis, C. L., Dauenhauer D., Humphrey J. M., Chamberlin A. R., Buller A., Monaghan D. T. and Bridges R. J. (1997). Methylation of the NMDA receptor agonist L-trans-2,3-pyrrolidine-dicarboxylate: enhanced excitotoxic potency and selectivity. *Tox. App. Pharm.* **144**: 45-55.

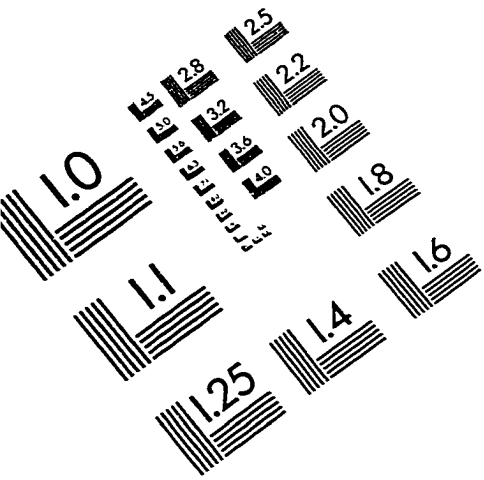
Willis, C. L., Humphrey J. M., Koch H. P., Hart J. A., Blakely T., Ralston L., Baker C. A., Shim S., Kadri M., Chamberlin A. R., et al. (1996). L-trans-2,3-pyrrolidine-dicarboxylate: characterization of a novel excitotoxin. *Neuropharm.* **35**(5): 531-539.

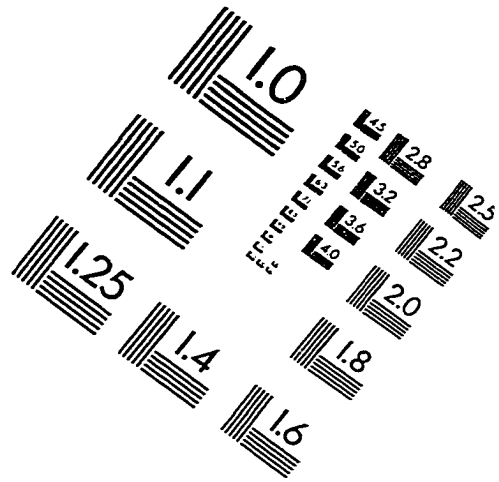
Yamashita, H., Kawakami H., Zhang Y., Hagiwara T., Tanaka K. and Nakamura S. (1995). Inhibition by folded isomers of L-2-(carboxycyclopropyl)glycine of glutamate uptake via the human glutamate transporter hGluT-1. *Eur. J. Pharm.* **289**: 387-390.

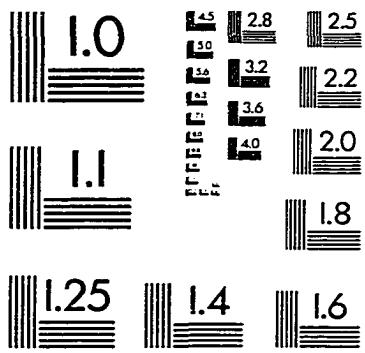
Zeevalk, G. D. and Nicklas W. J. (1991). Mechanisms underlying initiation of excitotoxicity associated with metabolic inhibition. *J. Phar. Expt. Ther.* **257**(2): 870-878.

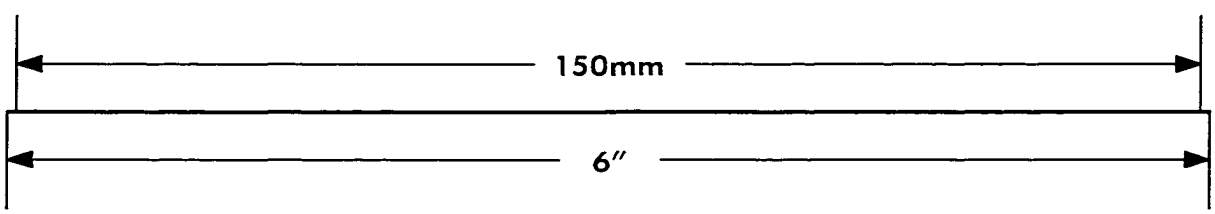
Zerangue, N. and Kavanaugh M. P. (1996). Flux coupling in a neuronal glutamate transporter. *Nature* **383**: 634-637.

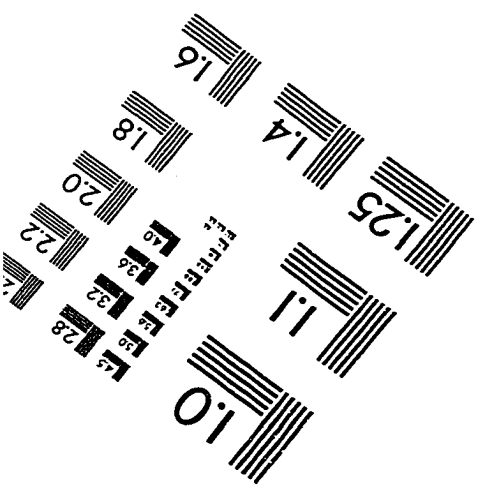
IMAGE EVALUATION
TEST TARGET (QA-3)

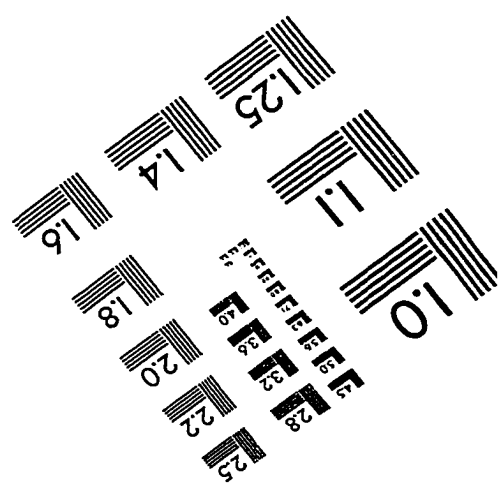












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989