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Pharmacological Modeling and Regulation of Excitatory Amino Acid Transporters (EAATs)

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PHARMACOLOGICAL MODELING AND REGULATION OF EXCITATORY AMINO ACID TRANSPORTERS (EAATS)

By

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L-Glutamate is the major excitatory neurotransmitter in the mammalian CNS that can mechanistically contribute to either neuronal signaling or neuronal pathology. Consequently, its concentration in the CNS must be carefully regulated, a critical need that is met by the excitatory amino acid transporters (EAATs). The presence of at least five isoforms of EAATs raises interesting questions as to potential structural and functional differences among the subtypes. We have investigated possible differences in the ligand binding domains of the EAATs through the development of computationally based pharmacophore models. An EAAT2-specific model was created with four potent and selective ligands that act as non-substrate inhibitors: *cis*-5-methyl-L-*trans*-2,3-pyrrolidine dicarboxylate, L-*anti*-*endo*-3,4-methano-pyrrolidine-3,4-dicarboxylate (L-*anti*-*endo*-3,4-MPDC), (2S,3R,4S)-2-(carboxy-cyclopropyl) glycine (L-CCG-IV) and L-β-*threo*-benzyloxy-aspartate (L-β-TBOA). This model predicts distinct regions that might influence the potency and selectivity of EAAT2 ligands, including: 1) a highly conserved positioning of the two carboxylate and the amino groups, 2) a nearby region that can accommodate selective modifications (e.g., cyclopropyl ring, CH_3 groups, and O atoms), and 3) a region occupied by the benzyl ring of L-β-TBOA. This model was also used in conjunction with L-β–*threo*-benzyl aspartate (L-β-TBA), a recently characterized preferential inhibitor of EAAT3, to identify possible differences between EAAT2 and EAAT3.

Functional studies on the EAATs also led to the identification of a putative modulatory mechanism that is specific for EAAT1. Thus, a series of sulfated neuroactive steroids, including pregnenolone sulfate (PREGS), were found to selectively increase the ability of EAAT1 to transport atypical substrates like D-aspartate and L-cysteine, but not L-glutamate. The effect was rapid, reversible, limited to a select group of sulfated steroids, and not observed with either EAAT2 or EAAT3. Interestingly, the action of PREGS could be blocked by the simultaneous addition of arachidonic acid, a previously recognized inhibitory modulator of EAAT1. The fact that this observed change in activity was produced by neurosteroids raises questions not only related to the regulatory mechanisms itself, but also to the possible role of neurosteroid in modulating glutamate transport.

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Chapter 1: Background and Significance:

The amino acid L-glutamate was first shown to excite spinal neurons in 1959 (Curtis et al., 1959). Since that time it has been shown to act in all the regions of the brain and is now accepted as the major excitatory amino acid neurotransmitter in mammalian CNS. Through activation of various ionotropic and metabotropic receptors in the CNS (Monaghan et al., 1989; Nakanishi et al., 1998; Cotman et al., 1987; Wollmuth and Sobolevsky, 2004), L-glutamate participates in most aspects of normal brain function including fast synaptic transmission, cognition, memory and learning (Fonnum, 1984; Collingridge and Lester, 1989; Hollmann and Heinemann, 1994; Mayer and Armstrong, 2004).

The concentration of L-glutamate in the brain is estimated to be 5-15 mmol per kg weight depending on the region (Schousboe, 1981; Krnjevic, 1970). However, the highest concentrations are found inside nerve terminals (~ 10 mM) (Ottersen et al., 1992; Ottersen et al., 1996) and the estimates of the concentration of glutamate in synaptic vesicles range from 60-210mM (Nicholls and Attwell, 1990; Burger et al., 1989; Riveros et al., 1986). The concentrations in extracellular fluid and in the cerebrospinal fluid are typically reported to be between 3 and 10 μM (Hamberger and Nystrom, 1984; Lehmann et al., 1983). This means that the concentration gradient of glutamate across the plasma membranes is at least several thousand-fold (Storm-Mathisen et al., 1983; Ottersen et al., 1992; Ottersen et al., 1996). Moreover, low micromolar concentrations of glutamate have the ability to activate both ionotropic receptors (Patneau and Mayer, 1990; Curras and Dingledine, 1992) and metabotropic receptors (Schoepp et al., 1999). The average

concentration of glutamate attained in the cleft following synaptic release has been estimated to be about 3mM (Clements, 1996; Diamond and Jahr, 1997; Harris and Sultan, 1995) which is enough to saturate and activate postsynaptic receptors at hippocampal synapses (Clements et al., 1992). Excessive activation of glutamate receptors can be neurotoxic, a phenomenon termed excitotoxicity (Olney, 1990), (Choi and Rothman, 1990; Choi, 1992; Meldrum, 1993; Doble, 1999). Disruptions in glutamate homeostasis have been implicated in various pathologic conditions, including: epilepsy, ALS, dementia, ischemia, brain and spinal cord injuries and hypoglycemia (Doble, 1999). Given both the excitatory and excitotoxic properties of L-glutamate, it is not surprising that its concentration within the CNS must be carefully regulated. This activity is ascribed to glutamate transporters found on both the neurons and glia (Gegelashvili and Schousboe, 1997; Kanai and Hediger, 2003).

Although glutamate is present in all cells, its release through exocytosis requires active transport into secretory vesicles. Three subtypes of vesicular glutamate transporters (VGLUTs) have been identified that concentrate glutamate into neurosecretory vesicles for regulated release. VGLUTs belong to SLC17A group (Reimer and Edwards, 2004) and includes VGLUT1 (SLC17A6), VGLUT2 (SLC17A7) and VGLUT3 (SLC17A8) (Bellocchio et al., 2000; Takamori et al., 2002; Takamori et al., 2000; Takamori et al., 2001; Aihara et al., 2000; Fremeau et al., 2001; Kaneko and Fujiyama, 2002). These transmembrane proteins are thought to have 6-12 predicted transmembrane domains. VGLUT activity is coupled to the proton electrochemical gradient ($\Delta\mu_{H+}$) generated by a vacuolar type H^+-ATPase (Moriyama et al., 1992; Bellocchio et al., 2000; Forgac, 2000).

VGLUTs transport glutamate with an affinity (K_m ~ 1mM) that is 100- to 1000-fold lower than that of the high affinity glutamate transporters present on the plasma membrane. Additionally, in contrast to the plasma membrane glutamate uptake, these vesicular counterparts do not transport aspartate. Vesicular transporters have a biphasic dependence on Cl^-, such that low concentrations activate uptake while high concentrations are inhibitory (Reimer et al., 2001; Shigeri et al., 2004).

Glutamate Receptors:

Glutamate receptors can be broadly divided into two major classes, ionotropic and metabotropic. The ionotropic receptors (iGluRs), also called ligand-gated ion channels (LGIC), are intrinsic transmembrane ion channels that open in response to the binding of a chemical messenger (in this case, glutamate). They are responsible for rapid signaling and produce relatively large conductance changes. On the other hand, the metabotropic glutamate receptors (mGluRs) belong to G protein-coupled receptor (GPCR) family as their signal is produced via guanine nucleotide-binding protein (or G-protein) linked to second messenger systems.

Ionotropic Glutamate Receptors: Three major subtypes of ionotropic glutamate receptors have been identified and named after the selective agonists that were used to pharmacologically distinguish them from one another (Monaghan et al., 1987; Collingridge et al., 1989). They include NMDA (N-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and KA (kainate), receptors. The binding of glutamate to the ionotropic receptors produces an excitatory postsynaptic

potential as a consequence of the opening of glutamate-gated ion channels permeable to both Na^+ and K^+ (Nicholls and Attwell, 1990), (Hosli et al., 1976). NMDA receptors are selectively blocked by the drug APV (2-amino-5-phosphonovaleric acid). The AMPA and kainate receptors are not affected by APV, but both are blocked by the dihydroquinoxaline derivatives like 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6,7-dinitroquinoxaline-2,3-dione (DNQX) (Kew and Kemp, 2005). Thus, the AMPA and kainate receptors have historically been referred to as non-NMDA receptors.

The ionotropic glutamate receptors are complexes of four subunits. Each subunit has a ligand-binding domain and possesses four membrane-associated segments (3 transmembrane and 1 reentrant loop). The channel-lining TM2 segment forms a loop and reexits into the cytoplasm (Wo and Oswald, 1995), (Wood et al., 1995).

NMDA receptor: NMDA-type channels open and close relatively slowly in response to glutamate and thereby contribute to the late phase of the EPSP (Hestrin et al., 1990). The NMDA receptor contains a cation channel that is permeable to Na^+, Ca^{2+} and K^+ (Hosli et al., 1976; Dingledine et al., 1999; Mayer and Armstrong, 2004). In particular, the Ca^{2+}-premeability for NMDA receptors appears to be much higher than that of non-NMDA glutamate receptor subtypes (McBain and Mayer, 1994) and other cation-selective receptors (Rogers and Dani, 1995).

NMDA receptor channels are obligate heterotetramers requiring NR1 and NR2 subunits to form functional channels. NR3 might also form functional channels with NR1, but it most commonly co-assembles with NR1 and NR2 to form complexes with unique

properties and NR3 subunit substitutes for one of the NR2 subunits. At least eight splice variants have been identified for the NMDAR1 subunit and these variants produce differences in the properties of the expressed receptor (Hollmann and Heinemann, 1994). Four other members of the NMDA receptor family have been cloned (NMDAR2A-2D) and their deduced primary structures are highly related. These four NMDA receptor subunits do not form channels when expressed singly or in combination unless they are co expressed with NMDAR1. Apparently, NMDAR1 contributes to the formation of an essential functional pore by which activation of NMDA occurs, while NMDAR2 receptors 2A-2D play important roles in modulating the receptor activity when mixed as heteromeric forms with NMDAR1 (Dingledine et al., 1999).

Various pharmacologically distinct sites that alter the activity of NMDA receptors have been characterized. These include:

1) a glutamate binding site that promotes the opening of a high-conductance channel that permits entry of Na^{+} and Ca^{2+} into target cells (Dingledine et al., 1999; Mayer and Armstrong, 2004; Hosli et al., 1976).
2) a strychnine-insensitive glycine-modulatory site. Activation of NMDA receptor channels requires binding of both L-glutamate and the co-agonist glycine. (Kleckner and Dingledine, 1988; Johnson and Ascher, 1987). Stoichiometrically, two molecules of NMDA and two molecules of glycine must bind to the NMDA receptor for activation of ion channel gating in *in vitro*, mouse hippocampal neurons (Benveniste and Mayer, 1991). Whereas, glycine appears to bind at distinct regions of the NR1 subunit, glutamate

binding resides in the homologous region of the NR2A and 2B subunits. Thus, agonist and coagonist binding sites are located on corresponding regions of distinct subunits of NMDA receptor channels. The crystal structure of the NMDA ligand-binding core of NR2A bound to glutamate and that of the NR1-NR2A heterodimer bound to glycine and glutamate confirms that these receptors are heteromeric ion channels that for activation require binding of glycine and glutamate to the NR1 and NR2 subunits respectively (Furukawa et al., 2005).

3) use-dependent PCP (phencyclidine) site (also binds MK801, ketamine) which act most effectively when the receptor is activated (Lerma et al., 1991; MacDonald et al., 1991).
4) voltage-dependent Mg^{2+}-binding site. The opening of NMDA receptor channel depends on membrane voltage as well as transmitter (Nowak et al., 1984; Mayer et al., 1984).
5) an inhibitory Zn^{2+} site produces voltage-independent block (Christine and Choi, 1990; Legendre and Westbrook, 1990).
6) polyamine-regulatory site whose activation by spermine and spermidine facilitates NMDA receptor-mediated transmission (Rock and MacDonald, 1992).

The significance of NMDA receptor is evident from its involvement in a wide range of neurophysiological and pathological processes such as memory acquisition, developmental plasticity, epilepsy and the neurotoxic effects of brain ischemia. Normal

levels of NMDA receptor activity are needed to promote survival and render neurons resistant to subsequent trauma (Hardingham and Bading, 2003). Excessive activation of NMDA receptors can lead to excitotoxic trauma and subsequently, neuronal death. The mechanism of cell death (apoptosis versus necrosis) is believed to depend on the severity of the insult. Rapid, necrotic cell death occurs after acutely excessive NMDA receptor activation. Slower apoptotic cell death occurs after a milder (although ultimately toxic) episode of NMDA receptor activation (Hardingham and Bading, 2003).

AMPA receptor: AMPA receptors are composed of four members (GluR1-4) that are products of separate genes. Like NMDAR, AMPAR is a tetramer of independent subunits (Rosenmund et al., 1998). Four glutamate molecules bind to activate these receptors (Rosenmund et al., 1998; Wollmuth and Sobolevsky, 2004). The AMPA receptor subunits can form either functional homomeric or heteromeric channels. The GluR2 subunit plays a critical role in determination of the permeability of heteromeric receptors to Ca^{2+}. Thus, AMPA receptors that do not contain GluR2 are Ca^{2+} permeable (Hollmann and Heinemann, 1994; Washburn et al., 1997). AMPA and quisqualate are preferred agonists whereas 2,3-dihydro-6-nitro-7-sulfamoylbenzo quinoxaline (NBQX) (Kew and Kemp, 2005) and 2,3-benzodiazepine derivatives (e.g., GYKI-52466) (Bleakman et al., 1996; Rogawski, 1993) appear to be selective antagonists at AMPA receptors.

Kainate Receptors: Kainate receptors are composed of two related subunit families, GluR5-7 and KA1 and 2. KA1 and 2 combine in heteromeric assemblies with members of the GluR5-7 subfamily to form functional receptors. The KA1 and KA2 homomeric

complexes have been shown to be non-functional. However, GluR5, 6 and 7 subunits can form functional homomeric receptors. Kainate receptor forms a tetrameric complex and is activated following the binding of four glutamate molecules (Rosenmund et al., 1998; Wollmuth and Sobolevsky, 2004). With its nearly ubiquitous expression in the brain, the KA2 subunit is likely a constituent of most neuronal kainate receptors (Swanson et al., 2002). Domoate and kainate are preferred agonists for these receptors. Topiramate, an anticonvulsant drug, has been shown to reduce seizures induced by kainic acid but not by AMPA (Perucca, 1997; Conti et al., 2002).

Metabotropic Glutamate Receptors:

The metabotropic glutamate receptors can be selectively activated by *trans*-(1S, 3R)-1-amino-1,3-cyclopentane dicarboxylic acid (ACPD). The action of glutamate on the ionotropic receptors is always excitatory, while activation of the metabotropic receptors can produce either excitation or inhibition. The widespread distribution of metabotropic receptors in the CNS coupled with the prevalence of glutamate as a neurotransmitter indicates that this system is a major modulator of second messengers in the mammalian CNS (Kew and Kemp, 2005).

The mGlu receptors are divided into three major groups on the basis of sequence homology, pharmacological profile, and second messenger coupling (Schoepp et al., 1999),(Kew and Kemp, 2005). Molecular cloning studies have revealed the existence of at least eight different subtypes of mGluR, mGluR1-mGluR8, which have a common

structure of a large extracellular domain preceded by the seven-membrane spanning domains.

Group I (mGluR1 and mGluR5) is coupled to stimulation of phosphotidylinositol hydrolysis/ Ca^{2+} signal transduction. Group II (mGluR2 and mGluR3) is negatively coupled through adenylyl cyclase to cyclic adenosine monophosphate formation. Group III (mGluR4, mGluR6-8) is also negatively linked to adenylyl cyclase activity but shows a different agonist preference from that of mGluR2 and 3. L-AP-4 (L-2-amino-4-phosphonobutyrate) is a potent agonist of mGluR4, 6, 7 and 8 but has little effect on other receptor subtypes. These receptors are shown to be involved in physiological and pathological conditions such as synaptic plasticity (Endoh, 2004; Bonsi et al., 2005), neurotoxicity and neuroprotection (Baskys et al., 2005), and drug addiction (Robbe et al., 2002).

Glutamate Transporters:

The excitatory signal is terminated by the high-affinity uptake of glutamate from the synapse by the glutamate transporters present in both astrocytes and neurons. In astrocytes, glutamate is taken up from the extracellular fluid and converted to glutamine by astrocyte-specific enzyme glutamine synthetase (GS) (Martinez-Hernandez et al., 1977). Glutamine has been reported to be critical for the maintenance of a normal level of glutamate in nerve terminals (Laake et al., 1995). Glutamine is released in the extracellular fluid, taken up by neurons and reconverted to glutamate by the phosphate-dependent mitochondrial enzyme glutaminase (Hertz et al., 1999; Magistretti et al., 1999;

Broer and Brookes, 2001; Kvamme et al., 2001). This metabolic pathway is referred to as 'glutamate-glutamine cycle'.

Glutamate Transporter Family: High-affinity sodium dependent glutamate transporters belong to the solute carrier family 1 (SLC1) that includes five eukaryotic glutamate transporters and two eukaryotic neutral amino acid uptake systems (Kanai and Hediger, 2003; Danbolt, 2001; Slotboom et al., 1999). The glutamate transporters contain between 500 – 600 amino acid residues (~65 kDa).

The neurotransmitter transporters are all ion-coupled carriers that mediate the accumulation of the neurotransmitter substrate using the movement of one or more ions down their concentration gradients. While these transporters are not directly coupled to the hydrolysis of ATP, they are indirectly coupled through the ion gradients generated by ion-pumping ATPases (Glynn and Karlish, 1975; Lingrel and Kuntzweiler, 1994). Thus, the plasma membrane transporters are indirectly driven by the Na^+/K^+ ATPase (Broer, 2002; Palacin et al., 1998) that generates gradients of Na^+ (out > in) and K^+ (in > out) and in the process creates a membrane potential ($\Delta\psi$, inside negative).

Initially, three glutamate transporters were identified by molecular cloning in 1992 in different laboratories at almost the same time. The L-glutamate/ L-aspartate transporter (GLAST) was isolated from the rat brain cDNA library (Storck et al., 1992). *In situ* hybridization revealed a high-density of GLAST mRNA in the Purkinje cell layer of cerebellum and less dense distribution throughout the cerebrum (Storck et al., 1992).

Glutamate transporter (GLT-1) was isolated from rat brain (Pines G, 1992). More recently, pharmacologically indistinguishable splice variants of GLT-1, referred to, as GLT-1b and GLT-1c have been isolated from rat forebrain and retina respectively (Chen et al., 2002; Rauen et al., 2004). EAAC1 was isolated from rabbit intestine using an expression cloning approach (Kanai, 1992). Subsequently, utilizing molecular cloning and functional expression, three homologous glutamate transporters, EAAT1 (GLAST / SLC1A3), EAAT3 (GLT-1 / SLC1A2) and EAAT3 (EAAC1 / SLC1A1), were isolated from human motor cortex (Arriza et al., 1994). Later, EAAT4 (SLC1A6) was isolated from the cerebellum and showed 65%, 41% and 48% amino-acid identity to the human glutamate transporters EAAT1, 2 and 3 respectively (Fairman, 1995). Screening a human retinal cDNA library has lead to the identification of EAAT5 (SLC1A7) (Arriza et al., 1997). Additionally, the human counterparts of GLT-1b and GLT-1c have also been identified from human brain and retina respectively (Lauriat et al., 2007; Rauen et al., 2004). The different EAAT subtypes exhibit 44-55% amino acid sequence identity with each other (Kanai and MA, 2003). For the purpose of clarity the EAAT nomenclature will be used throughout this dissertation.

Among the five glutamate transporter subtypes, EAAT2 (GLT-1) has been shown to be the major transporter and is responsible for over 90% of glutamate uptake in the rat forebrain (Haugeto et al., 1996; Tanaka et al., 1997).

EAAT Localization: EAAT2 (GLT-1) and EAAT1 (GLAST) are considered to be primarily localized to astroglia. The EAAT2 (GLT-1) protein has been found in

astrocytes in the normal adult rat brain and spinal cord (Rothstein et al., 1994; Chaudhry et al., 1995; Lehre et al., 1995; Ullensvang et al., 1997; Berger and Hediger, 2000). Also, there are reports suggesting the expression of EAAT2 (GLT-1) in hippocampal neurons (Chen et al., 2004). While EAAT2 (GLT-1) is abundant in forebrain, particularly in hippocampus, lateral septum, cerebral cortex and striatum (Danbolt et al., 1992; Haugeto et al., 1996; Tanaka et al., 1997), relatively lower levels are expressed in the cerebellum (Lehre et al., 1995). Using RT-PCR and northern blotting, EAAT2b (GLT1b) was shown to be expressed in various regions of the rat and human brain, including: amygdala, hippocampus, nucleus accumbens and prefrontal cortex (Lauriat et al., 2007). The third isoform, EAAT2c (GLT1c) and its human counterpart were found to be mostly expressed in retina (Rauen et al., 2004).

EAAT1 (GLAST) is considered the major glutamate transporter in the cerebellum (Lehre and Danbolt, 1998), the inner ear (Furness and Lehre, 1997), the circumventricular organs (Berger and Hediger, 2000), and in the retina (Rauen, 2000). EAAT1 is most abundant in Bergmann glia in the brain cerebellar molecular layer, but is also present in the cortex, hippocampus and deep cerebellar nuclei. EAAT1 is expressed throughout the CNS, but in different amounts in different regions. EAAT1 is expressed primarily in astroglial cells (Rothstein et al., 1995; Chaudhry et al., 1995; Lehre et al., 1995; Schmitt et al., 1997). EAAT1 (GLAST) and EAAT2 (GLT-1) have been shown to be expressed by the same astrocytes (Lehre et al., 1995; Haugeto et al., 1996) but in different proportions throughout the brain (Lehre et al., 1995) and coexist in the same astroglial cell membranes as separate homo-oligomeric complexes (Haugeto et al., 1996).

However, GLT and GLAST have also been observed in morphologically distinct astrocytes in primary hippocampal cultures (Perego et al., 2000).

EAAT3 (EAAC1) is mostly expressed in neurons, such as large pyramidal cortical neurons and Purkinje cells, but does not appear to be necessarily selective for glutamatergic neurons. It is highly enriched in the cortex, hippocampus and caudate-putamen and is confined to pre- and postsynaptic elements (Kanai, 1992; Rothstein et al., 1994; Conti et al., 1998; Kugler and Schmitt, 1999). However, some astroglial expression has been reported (Conti et al., 1998; Kugler and Schmitt, 1999).

EAAT4 is most abundant in the Purkinje cells of the cerebellar molecular layer in adult CNS of the rat (Nagao et al., 1997; Dehnes et al., 1998) and human (Fairman, 1995; Furuta et al., 1997; Bar-Peled et al., 1997; Otis and Jahr, 1998).

While EAAT5 shows strong signal in retina, its expression in the brain has not been detected (Arriza et al., 1997; Eliasof et al., 1998a; Eliasof et al., 1998b).

Stoichiometry: Glutamate transport across the plasma membrane is coupled with the movement of the inorganic ions, thus, utilizing the free energy stored in their electrochemical gradients. Three sodium (Na^+) ions and a proton are co-transported with each molecule of glutamate while one potassium (K^+) ion is counter-transported for EAAT3 (Zerangue and Kavanaugh, 1996a), EAAT2 (Levy et al., 1998) and EAAT1 (Owe et al., 2006). Thus, the thermodynamic estimates from these studies indicate that

EAATs could lower extracellular glutamate to a low nanomolar range (Zerangue and Kavanaugh, 1996a; Levy et al., 1998; Owe et al., 2006). The transporter also produces a chloride current that is activated by glutamate, but is not stoichiometrically coupled to glutamate transport. This anion flux follows its own transmembrane electrochemical potential gradient (Fairman, 1995; Wadiche et al., 1995; Eliasof and Jahr, 1996; Billups et al., 1996). The importance of the anion channel is beginning to be recognized and EAAT5 has been shown to exhibit autoreceptor properties caused by hyperpolarization by chloride ions in the retinal bipolar cells (Veruki et al., 2006).

Mechanisms and Structure: Uptake through the EAATs is thought to proceed through an "alternate-access" mechanism (Jardetzky, 1966; DeFelice, 2004; Yernool et al., 2004; Boudker et al., 2007; Koch and Larsson, 2005). The binding of extracellular glutamate and required Na^+ ions causes conformational change that exposes the substrate and ions to intracellular milieu where they are released. Subsequently, binding of intracellular K^+ ion reverts the transporter back to extracellularly open state. Accordingly, binding-sites for glutamate and ions alternately face extracellular or cytoplasmic compartments. Within this framework, coupling results from conformational changes induced by substrate and ions binding (Grunewald et al., 1998; Grunewald and Kanner, 2000; Slotboom et al., 1999; Slotboom et al., 2001; Zarbiv et al., 1998; Seal and Amara, 1998; Seal et al., 2000). However, a clear distinction between the "rocker-switch" model (Abramson et al., 2003; Huang et al., 2003) and "two-gated" channel model (Lester et al., 1996; Lester et al., 1994; Cao et al., 1998; Larsson et al., 2004) has yet to be resolved. Based upon the cysteine-scanning accessibility studies of mammalian glutamate transporters as well as

from the 3.5Å crystal structure of archeael EAAT homologue GLT_{PH}, it appears that these transporters possess eight transmembrane domains along with two oppositely oriented reentrant hairpin loops (HP1 and HP2) (Grunewald and Kanner, 2000; Yernool et al., 2004; Seal et al., 2000). Earlier suggestions that the binding of substrate and required Na^+ ions causes conformational changes in glutamate transporters to result in transport of the substrate (Brocke et al., 2002; Slotboom et al., 1999) was reinforced by the recently available crystal structure (Boudker et al., 2007). The suggestion by Boudker et al., that the extracellularly accessible HP2 loop forms the extracellular gate is compelling. This model suggests that the binding of the substrate and Na^+ ions leads to closing of HP2 loop and subsequent transport, while binding of non substrate like L-β-TBOA prevents HP2 from closing and locks the transporter in an "open" state.

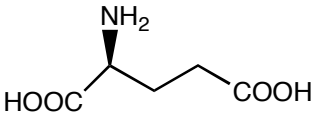
EAAT glutamate transporters assemble as homotrimeric complexes (Koch and Larsson, 2005; Gendreau et al., 2004; Yernool et al., 2004; Boudker et al., 2007; Haugeto et al., 1996). Despite the multimeric nature of the complex, the subunits seem to act independently (Koch and Larsson, 2005; Koch et al., 2007; Leary et al., 2007; Grewer and Rauen, 2005). However, some results suggest that glutamate carriers may interact cooperatively during anion channel activation (Torres-Salazar and Fahlke, 2006). The significance of the multimeric nature of glutamate transporters is still unclear. It is likely that this assembly is important for the cell-surface expression of functional transporters. It has also been suggested that the hydrophilic surface of the bowl formed by the assembly of the subunits may aid in the transport of charged solute (Kavanaugh, 2004).

EAAT Pharmacology: The presence of five glutamate transporters raises an obvious question as to what specific physiological role each might play in defining the proper functioning of the brain. An especially fruitful strategy to establish the roles of different protein targets has been to pharmacologically block the corresponding proteins using selective inhibitors and analyze the resulting effects. This approach is dependent upon the development of subtype-selective inhibitors and substrate; which in turn, rely on a thorough understanding of the structure-activity relationships (SAR) that govern binding and uptake by the various EAATs. With this concept in mind, we have generated an EAAT2 binding site-specific pharmacophore model exploiting the various features of the diverse and relatively specific EAAT2 inhibitor profiles.

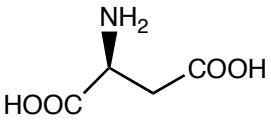
Along with L-glutamate, numerous ligands have been identified as potent, competitive inhibitors of the EAATs (Bridges et al., 1999). Most of these identified ligands are found to be α-amino acids and possess the second carboxylate group that is located 2-3 carbon atoms away from the proximal carboxylate (Figure 1.1). The distance between the two carboxylates seems to be critical. For instance, L-α-aminoadipic acid (L-α-AA), which contains longer chain length, has been shown to be a poor inhibitor of glutamate uptake at EAAT1, 2 and 3 (Arriza et al., 1994). Both, L-aspartate and D-aspartate were found to be effective substrates in oocytes expressing individual glutamate transporters (Arriza et al., 1994; Arriza et al., 1997; Fairman, 1995). However, D-glutamate was found to have negligible activity as an inhibitor of EAATs. Thus, the stereochemistry at the α-carbon of glutamate appears to be an important criterion for activity, although the requirement may vary among analogues.

Among the simple modifications made in early SAR studies, some changes to the distal carboxylate seem to be acceptable. For example, the inhibitory activity is retained for compound like L-serine-O-sulfate, cysteic acid and cysteine sulfinic acid, in which the distal carboxylate of L-aspartate is replaced by sulfonate or sulfinic groups. However, the phosphonic acid analogue, AP4, has been reported to exhibit no activity at the EAATs (Bridges et al., 1999). Whereas L-β-TBOA blocks all subtypes, compounds like dihydrokainate (DHK) and L-*trans*-2,3-PDC can selectively block EAAT2. A detailed pharmacology is presented in the introduction to Chapter 3 of this research report. The available structure-activity information is most advanced for EAAT2 ligands and therefore a more extensive study has been undertaken in the present research to predict the structural characteristics that define both the potency and specificity for this particular transporter subtype. This was done by utilizing four relatively EAAT2-specific inhibitors in the training set to build the pharmacophore model. The validity of this model was confirmed by analyzing three additional inhibitors. This robust model predicted the unique binding-site characteristics. Further, the relatively selective EAAT3 inhibitor, L-β-*threo*-BA is aligned with this model to delineate the differences in the binding-sites for EAAT2 and EAAT3 (Esslinger et al., 2005). A detailed account is presented in Chapter 3 of this dissertation.

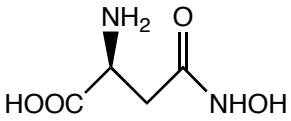
Figure 1.1. Simple glutamate analogues.



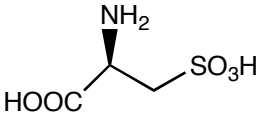
L-Glutamic acid



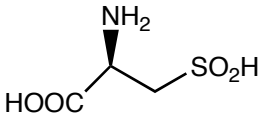
L-Aspartic acid



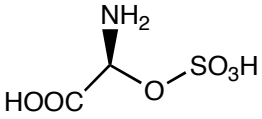
L-Aspartate-β-hydroxamate



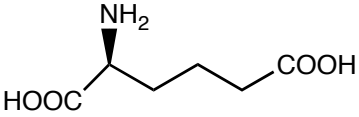
L-Cysteic acid



L-cysteine sulfinic acid



L-Serine-O-sulfate



L-α-Aminoadipic acid

Glutamate Transporter Dysfunction: Functional losses of the glutamate uptake system can lead to cellular dysfunction and neurotoxicity either by direct action or participation in a cascade of disruptive cellular events (Maragakis and Rothstein, 2001; Maragakis and Rothstein, 2004). A number of pathophysiological conditions such as ALS (Rothstein et al., 1995; Rothstein et al., 1992; Van Den Bosch et al., 2006), Alzheimer's disease (Masliah et al., 1996; Masliah et al., 2000), Huntington's disease (Lievens et al., 2001), stroke/ischemia (Rossi et al., 2000), epilepsy (Rothstein, 1996; Tanaka et al., 1997) and schizophrenia (Ohnuma et al., 2000) have been associated with abnormal functioning of glutamate transporters.

Regulation of EAATs: Glutamate-mediated neurotransmission is believed to involve not only the synaptic but also extrasynaptic receptor activation. Synaptically released glutamate has the potential to diffuse into extrasynaptic space (Scanziani et al., 1997; Mitchell and Silver, 2000) as well as nearby synapses (DiGregorio et al., 2002). This "spillover" of glutamate contributes to neurotransmission by acting at mGluRs and iGluRs on both pre- and post-synaptic neurons (Brasnjo and Otis, 2001). The spillover of glutamate has been shown to inhibit GABA release from Golgi cell terminals (Mitchell and Silver, 2000) as well as glutamate release at mossy fiber synapse (Scanziani et al., 1997) by activating presynaptic mGluRs. Also, it has been shown to activate extrasynaptic NMDA receptors in mitral cells of olfactory bulb (Isaacson, 1999) and AMPA receptors present within adjacent synapses on the granule cells of the cerebellum (DiGregorio et al., 2002). The extent of extrasynaptic diffusion and the crosstalk between neighboring excitatory synapses may be markedly influenced by the location and activity

of glutamate transporters (Asztely et al., 1997). Therefore, strict regulation of glutamate concentration is of utmost importance for normal excitatory neurotransmission.

Glutamate uptake can be regulated by a number of different pathways, including: transcription and translation (Rothstein et al., 2005; O'Shea et al., 2006), trafficking (Gonzalez and Robinson, 2004; Hughes et al., 2004) as well as allosteric modification of the binding site (Vandenberg et al., 2004).

Transcription and Translation: Astrocytic EAAT2 (GLT-1) mRNA and protein are upregulated in astrocyte-neuron co-cultures via diffusible molecules secreted by neurons (Schlag et al., 1998). Dibutyryl cyclic adenosine monophosphate (dBcAMP) has been shown to elevate the EAAT2 (GLT-1) protein expression in both the membrane and cytoplasm of rat primary astrocytes (Swanson et al., 1997; Danbolt, 2001). The effects of dBcAMP on EAAT2 (GLT-1) expression in rat cortical astrocytes are mediated through protein kinase A and the MAP/Erk kinase pathway. Akt, also known as protein kinase B (PKB) induces the expression of EAAT2 (GLT-1) through increased transcription without affecting EAAT1 (GLAST) expression in astrocytes (Li et al., 2006).

EAAT expression can be regulated at the level of protein synthesis either via increased transcription of *glt1* gene (Su et al., 2003) or through increased translation of the EAAT2 transcript (Tian et al., 2007). Increased expression of *glt1* gene has been found with β-lactam antibiotic, ceftriaxone (Rothstein et al., 2005), and many extracellular factors, such as EGF (Zelenaia et al., 2000), injury-induced growth factors (EGF, TGFα, FGF-2

and PDGF) (Figiel et al., 2003; Schlüter et al., 2002), lipopolysaccharide (O'Shea et al., 2006). Additionally, several chemical entities, including corticosterone and retinol, have been identified that stimulate the translation of EAAT2 transcript (Tian et al., 2007).

Trafficking: In astrocyte-neuron co-cultures and C6 glioma cells expressing EAAT2 (GLT-1), activation of PKC by phorbol ester, phorbol 12-myristate 13-acetate (PMA 30′ pretreatment), causes a decrease in GLT-1 cell surface expression by interaction with the carboxyl-terminal domain (Kalandadze et al., 2002; Zhau and Sutherland, 2004). Unlike EAAT2 (GLT-1), EAAT3 (EAAC1) exists predominantly (>70%) in the intracellular compartment, but can be rapidly redistributed to the cell surface when stimulated by treatments such as platelet-derived growth factor or activation of PKC (Sims et al., 2000; Fournier et al., 2004; Davis et al., 1998). PMA treatment causes 80% increase in transporter activity within minutes in C6 glioma cells, which endogenously express only EAAT3 (EAAC1). Moreover, two different PKC isozymes increase EAAT3-mediated uptake by different mechanisms. PKC_{α} seems to selectively increase transporter cell surface expression. This effect is associated with redistribution of EAAT3 to the cell membrane and appears to be dependent in direct interaction of PKC_{α} with EAAT3 protein (Gonzalez et al., 2003). PKC_{ε} regulates uptake by a trafficking-independent mechanism, perhaps by increasing the intrinsic activity of the transporter in C6 glioma cells (Gonzalez et al., 2002). However, this modulation of EAAT3 (EAAC1) occurs in a cell-type specific fashion, since activation of PKC leads to opposite effects in EAAT3 (EAAC1) expressed in different culture models. It was shown that EAAT3 (EAAC1) expressed in *Xenopus* oocytes is downregulated by activation of protein kinase

C (Trotti et al., 2001). This downregulation was found to be associated with decrease in the maximal transport rate (V_{max}) and a movement of the transporter from the plasma membrane to the intracellular compartments, with no change in the affinity for glutamate. Another study also showed that activation of PKC induces a consistent decrease in the activity of EAAT3 expressed in the human astrocytoma cell line U373 (Dunlop et al., 1999). Acute treatment with PMA has been shown to cause an ~20% increase in transport by increasing catalytic efficiency/turnover number of EAAT1 (GLAST) (Susarla et al., 2004).

Allosteric Modification: Several compounds have been discovered that differentially effect glutamate transporter activity by allosteric modulation. These include the effects of arachidonic acid (Zerangue et al., 1995), zinc (Mitrovic et al., 2001), polyunsaturated fatty acids (Fairman et al., 1998) and EAAT3 interacting protein, GTRAP3-18 (Lin et al., 2001). Data presented in this work identify a putative modulatory site on EAAT1 that can increase activity. Interestingly, the compounds that revealed this potential regulatory site are all sulfated steroids.

Neuroactive Steroids: "Neuroactive steroids" is the general term that encompasses all the steroids present in the brain. These compounds may be derived by *in situ* synthesis, obtained from the peripheral hormones, or converted by enzymatic activation into active metabolites (Paul and Purdy, 1992; Melcangi and Panzica, 2006). Moreover, neuroactive steroids, e.g., pregnenolone (PREG), dehydroepiandrosterone (DHEA) and their sulfate

and lipoidal esters are present in higher concentrations in tissue from the nervous system (brain and peripheral nerves) than in the plasma (Baulieu, 1997).

In general, steroid effects can be divided into 'genomic' and 'non-genomic' mechanisms. The genomic effects are characterized by their delayed onset and prolonged duration while non-genomic effects are rapid in onset and short in duration (McEwen, 1994). In the latter case, steroids can produce immediate changes (within seconds) in neuronal excitability on a timescale that precludes a genomic locus of action. Progestins, estrogens, androgens, and corticosteroids are capable of modifying brain functions and behaviors by mechanisms that involve the classic genomic model for steroid action (McEwen et al., 1983).

The non-genomic effects of neuroactive steroids are produced mainly via their action on membrane proteins. The most thoroughly characterized membrane targets have been $GABA_A$, NMDA and σ receptors (Belelli and Lambert, 2005; Covey et al., 2001; Monnet and Maurice, 2006). In particular, progesterone derivatives like 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) and 3α-hydroxy-5β-pregnan-20-one (pregnanolone) are positive allosteric modulators of the γ-aminobutyric acid type A ($GABA_A$) receptor and negative modulators of NMDA receptors and are, therefore, considered inhibitory steroids (Belelli and Lambert, 2005; Lambert et al., 1995; Park-Chung et al., 1994; Park-Chung et al., 1997). Pregnenolone sulfate (PREGS) and dehydroepiandrosterone sulfate (DHEAS) are negative modulators of the $GABA_A$ receptor and positive modulators of the N-methyl-D-aspartate (NMDA) receptor and are therefore, categorized as excitatory

neurosteroids (Covey et al., 2001; Majewska, 1992; Wu et al., 1990; Bowlby, 1993). Additionally, the behavioral effects of PREGS and DHEAS, on memory associated with the NMDA receptor activity appear to be influenced by their pharmacological action at σ_1 sites (Monnet and Maurice, 2006; DeCoster et al., 1995; Lockhart et al., 1995; Maurice et al., 1997).

Pharmacotherapeutic potential of Neurosteroids: Changes in neurosteroid levels are associated with various physiological conditions, including: stress, pregnancy, neural development and ageing (Paul and Purdy, 1992; Schumacher et al., 2003). In addition to inducing anaesthesia, lower doses of steroid are found to produce anxiolytic, sedative and hypnotic effects (Gasior et al., 1999; Eser et al., 2006; Rupprecht, 2003; Goodchild et al., 2001). Data from preclinical and clinical studies support the potential efficacy of neuroactive steroids as a novel class of drugs for the therapeutic management of epilepsy, insomnia and drug dependence (Gasior et al., 1999; Rupprecht, 1997; Rupprecht et al., 1996; Gee et al., 1995).

The potential that neuroactive steroids modulate different aspects of glutamatergic signaling is a current focus of our lab. For example, Wes Smith in our lab has recently characterized the competitive inhibition of VGLUTs by these steroids (Smith Ph.D. dissertation, University of Montana). In the present work, we identify and characterize the effect of some neuroactive steroids on the glutamate transporter EAAT1 using C17.2 cells and rat primary astrocyte cultures. We find that neuroactive steroid such as pregnenolone sulfate (PREGS) can alter the transporter property of EAAT1.

SPECIFIC AIMS

Specific Aim 1: What structural characteristics of the ligands determine the selectivity and potency for EAAT2 (Excitatory Amino Acid Transporter 2) as determined by molecular modeling?

a. Generate and select the most promising pharmacophore models for EAAT2 using non-transportable inhibitors by employing various superpositioning possibilities.
b. Validate the pharmacophore models using the "leave-one-out" protocol. Superpositioning three additional non-transportable inhibitors of EAAT2 will test the robustness of the model. The acceptable model will generate satisfactory scoring function values for these superpositionings.
c. Incorporate the substrate ligands into the model (from 'a') to elucidate the structural and spatial features that may determine the binding and transport of the ligands by EAAT2
d. Superposition selective ligand for EAAT3 (viz., L-β-*threo*-benzyl aspartate) to delineate the points of divergence between EAAT2 and EAAT3.

Specific Aim 2: To determine the specificity with which the neurosteroids alter the uptake of ^{3}H-D-asparatate by EAATs

a. To test if the neurosteroids alter the ability of different EAAT subtypes to sequester D-[^{3}H]-aspartate.

b. To determine which other neurosteroids modulates the uptake of D-aspartate by EAAT1
c. To test if PREGS affect the uptake of D-aspartate by EAAT1 in different cell systems namely, C17.2 cells, primary astrocytes, oocytes and HEK293T cells over-expressing EAAT1.

Specific Aim 3: To investigate the mechanism by which PREGS alter the activity of EAAT1. The effect of the PREGS on the uptake of known substrates including L-glutamate, L-aspartate and D-aspartate will be described. Whether there are changes in the uptake kinetic parameters (such as K_m and V_{max}) of these substrates will also be evaluated.

Specific Aim 4: To determine the effect of PREGS on the ability of other EAAT1 ligands (both alternative substrates and non-substrates) to inhibit the uptake of D-aspartate and L-glutamate. Endogenous, as well as non-endogenous substrates and ligands of EAAT1 will be exploited.

Chapter 2: Methods and Materials

Molecular Modeling:

Computational work was performed on Silicon Graphics, Inc. (SGI) Octane workstations with R12,000 processors coupled to an SGI Origin 2000 server. The software application suite Sybyl (versions 6.8-7.3), with the Advanced Computation module (Tripos; St. Loius, MO), was used in adjunct with the industrially derived stochastic random search algorithm AESOP (Masek, 1998). In later versions of Sybyl (7.0 and up), Tripos dynamics was used instead of AESOP. Molecular databases were prepared in Sybyl formats. Data extracted from the molecular spreadsheets were occasionally exported for sorting and other manipulations in PERL and C code format that were automated. Inspections of conformations and Multifit superpositions were performed in Sybyl stereoview with CrystalEyes viewers.

The pharmacophore models were constructed as steric-strain, gas-phase derived compositions employing established comprehensive conformational analysis methods (Oprea et al., 1995; Marshall, 1995) with four EAAT2 inhibitor training set ligands, i.e. L-*anti*-*endo*-3,4-methanopyrrolidine-3,4-dicarboxylate (MPDC), *cis*-5-methyl-L-*trans*-2,3-PDC (PDC), (2S,3R,4S)-2-(carboxy-cyclopropyl)glycine (CCG-IV) , and L-β-*threo*-benzyloxy-aspartate (L-β-TBOA).

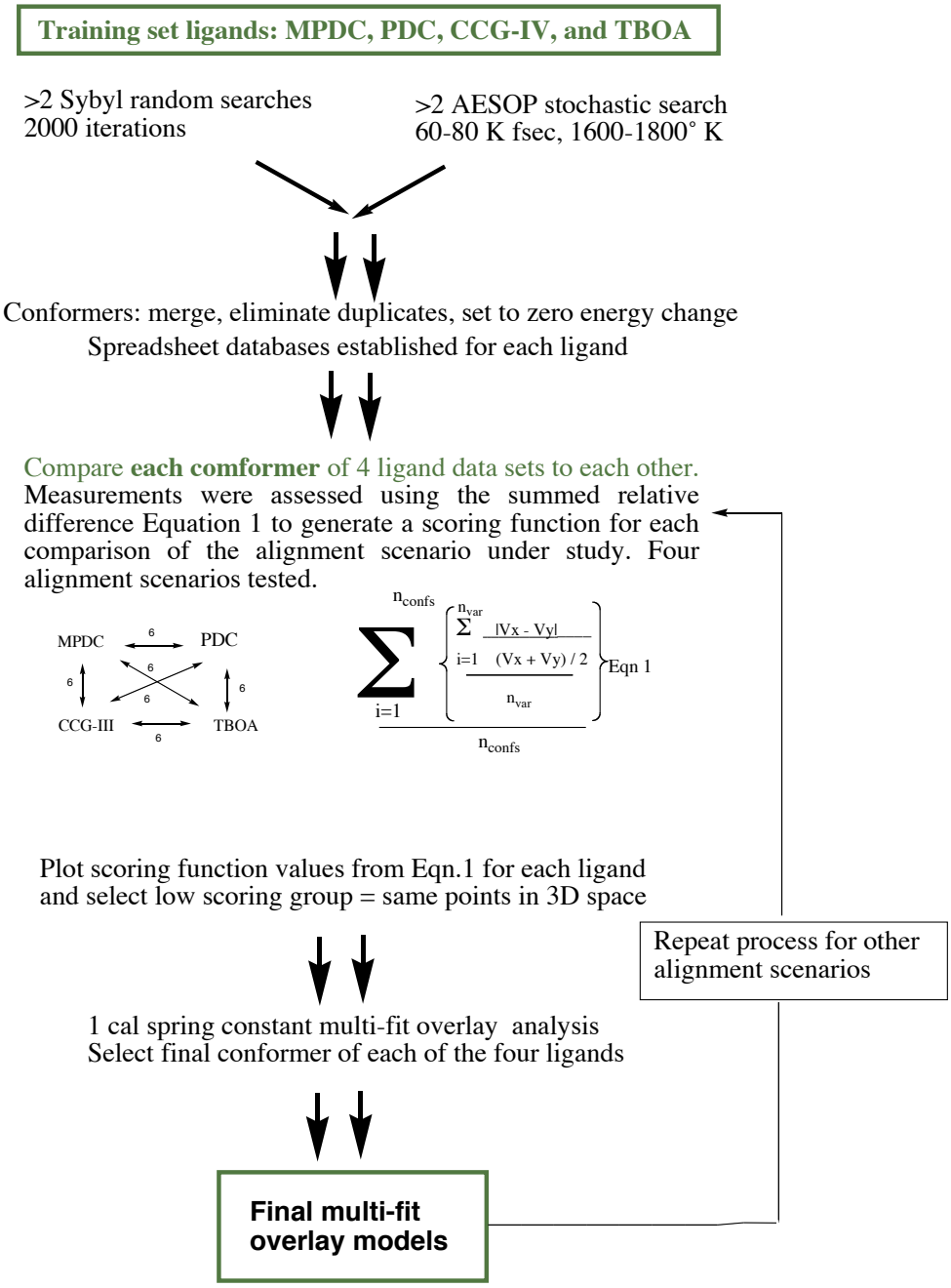
Conformational space of the EAAT inhibitors in the training and test sets was comprehensively searched employing two computational protocols: random search (Tripos Sybyl) and the stochastic technique AESOP (Masek, 1998). Because of the

random changes, these methods are able to access completely different region of the conformational space from one iteration step to the next i.e., it allows 'jumps' into high-energy region of the molecular hyperspace. This ensures a broad sampling of the conformational space. The random search procedure locates energy minima by randomly adjusting the selected bonds and minimizing the energy of the resulting geometry. Chiral centers, ring closure distances, and energy ranges were checked for consistency. This comparison was based on an RMS match between non-hydrogen atoms in the previously found conformers and the current conformer. At least two searches were performed on each training set ligand and other test cases. Data from the Sybyl random searches was deposited into a molecular database. AESOP (An Energy and Structure Optimization Protocol) and Tripos dynamics are alternative stochastic derived programs used to search conformational space. They apply high temperature to the molecule (which results in the molecule being torqued and tensed), and were set to capture a conformer snapshot every 5 femtoseconds. As the temperature falls, states of lower energy become more probable according to the Boltzmann distribution. Temperatures and times were set between 1600-1800 ^{0}K and 60-80K femtoseconds. Data from the AESOP and dynamics spreadsheets were deposited into the databases established earlier. Subsequently, all conformers from both search protocols were minimized to zero energy change defining their nearest energy well profile. Conformer database entries were sorted as a function of conformer total energy and cases of degenerate energy profiles were crosschecked as plausible duplicates, based on select distances and angles defined in an exported Molfile spreadsheet. Duplicate or nearly identical conformers (e.g., some non-essential rotamers

for L-β-TBOA) were eliminated. Some conformations would not have been found if only one conformer search routine had been used.

An extended closed form analysis method, which used the conformational data, was developed to select one conformer of each of the four ligands to form the 3D superposition models. To compare one conformation of one ligand to all conformations of each of the remaining three training set ligands, an all-combination comparison regimen was used by forming conformational comparison groups (shown as double headed arrows in Figure 2.1). For each comparison group, six distinct measures were assessed between each ligand conformer, thus permitting an assessment of molecular similarity. These six measures included the three distances and three angles between the proximal carboxylate carbon, C1; amino nitrogen, N and distal carboxylate carbon, C2 common to each training set ligand. Thus, the molecular spreadsheets included 3 angles and 3 distances, along with the energy (in kcal/mol), for each conformer per training set ligand. Additional molecular spreadsheets were also constructed in which *i*) the ether oxygen of L-β-TBOA and *ii)* the cyclopropyl centroid of L-CCG-IV were substituted for point C2 to consider alterative relative alignments. The resulting 3.4×10^6 conformational comparison groups were analyzed for 3D molecular similarity using a relative difference scoring function (Figure 2.1), defined as a sum of the average of the four conformations (as per the all-combination regime and denoted as $n_{conformers}$) using absolute value relative difference measurements ($n_{measures}$). Averaging precluded the use of weighting factors.

Figure 2.1. Computational paradigm used to generate the EAAT2 pharmacophore model.



For Eqn 1: V are 6 variable measures (3 distances and 3 angles) as compared for ligand x vs. ligand y; nvar = 6 (measures); nconfs = 6 as per comparison regime (double headed arrows).

The relative difference comparison measures between the conformers in a group (double headed arrows, Figure 2.1) included three distance and three angle values. The six conformer-to-conformer measurements were extracted from the molecular database spreadsheets and the calculations of the scoring functions were made. Conformer energies were not used in these calculations, thus making the scoring function energy independent. The comparison group that had a low scoring function value (least amount of differences amongst the six variable conformer measures in 3D space) was identified. Thus, the lower the score the more similar the conformers are to one another, representing molecular similarity of their space groupings. The selected ligand conformer set was brought together with a 1 cal spring constant, and the superposition models were appraised in stereoview using CrystalEyes viewers. The predictability quality of the model was assessed using a leave-one-out protocol (Marshall, 1995). The molecular spreadsheets containing all the conformers of test set ligand were exported into MS Excel spreadsheet and the similarity calculated using equation 1.

Modulation of EAATs by neuroactive steroids:

Materials: General cell culture supplies were purchased from Becton Dickinson (Franklin Lakes, NJ), Corning (Corning, NY), and Life Technologies (Grand Island, NY). D-[^{3}H]-Aspartic acid, L-[^{3}H]-glutamic acid and L-[^{3}H]-aspartic acid were purchased from Dupont NEN (Boston, MA). D,L-β-*threo*-Benzyloxy-aspartate was obtained from Tocris (Ballwin, MO). The steroids were purchased from Steraloids (Newport, RI). Remaining chemicals were obtained from Sigma (St. Louis, MO). FuGene 6 was purchased from Roche (Indianapolis, IN).

EAAT expression and cell culture: The glutamate transporter constructs for hEAAT1, hEAAT2 and hEAAT3 have been prepared and characterized by the other members of our lab previously (Esslinger et al., 2005). Briefly, EAAT1, and EAAT3 cDNA were PCR amplified from pBlueScript hEAAT1 and pBlueScript-hEAAT3 (provided by Dr M. Kavanaugh) using primer pairs (forward: 5′ATAAGGATCCATGACTAAAAGCAACGGA3′ and reverse: 5′TATTGATATCCTACATCTTGGTTTCACT3′) and (forward: 5′ATAAGGATCCATGGGGAAACCGGCGAGG3′ and reverse: 5′TATTGATATCCTAGAACTGTGAGGTCTG3′) respectively. Each primer pair introduced BamHI sites at the 5′ ends and EcoRV sites at the 3′ ends of each amplified fragment. The PCR fragments were then subcloned into the BamHI and EcoRV sites within the polylinker of the AAV vector pAM-CAG-WPRE (kindly provided by Dr Mathew During, University of Auckland, NZ) to create pAM-CAG-EAAT1-WPRE and pAM-CAG-EAAT3-WPRE. Final clones were confirmed by double stranded sequencing. A 1.9 kb EcoRI fragment containing the hEAAT2 cDNA clone was subcloned from pBlueScript-hEAAT2 (Dr M. Kavanaugh) into the EcoRI site of pAM-CAG-WPRE by standard molecular biology techniques to create pAM-CAG-EAAT2-WPRE. C17.2 cells (obtained from Dr Evan Snyder, Burnham Inst., La Jolla, CA) and HEK293T cells between passages 10 and 20 were seeded at 7×10^4 to 1×10^5 cells/well and 1.5×10^5 to 2×10^5 respectively in 12-well plates and grown in complete DMEM supplemented with 10% fetal bovine serum, 1mM sodium pyruvate, 0.1mM nonessential amino acids solution, and 0.05% penicillin / streptomycin (5000 units/ml) and gentamicin sulfate

(0.05 mg/ml). At 24 h after plating, cells were transfected using FuGene 6 or Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) in a ratio of 4 ml of Fugene 6 / Lipofectamine 2000 to 3 mg of purified plasmid DNA in accordance with the manufacturer's instructions. After 24 h, the relative levels of functional D-[^{3}H]-aspartate, L-[^{3}H]-glutamate or L-[^{3}H]-aspartate uptake were determined by the method of Martin and Shain (1979) as described below.

Primary astrocytes from rat cerebral cortex were prepared by following the protocol described by McCarthy et al (McCarthy, 1980). In brief, 2- to 4- day-old rat pups were decapitated and the cortices isolated in Ca^{2+}-Mg^{2+}-free buffer (CMF). CMF (in mM): HEPES 20, $NaHCO_3$ 4.2, Na^+ pyruvate 1, 1X HBSS and bovine serum albumin (3mg/ml). After gentle triturition, the dissociated cells were stored in DMEM/F12 medium supplemented with 15% FCS, 10mM HEPES, 14.28mM $NaHCO_3$, 0.5mM Na pyruvate, 0.05% penicillin / streptomycin (5000 units/ml) and gentamicin sulfate (0.05 mg/ml). After 24 hours, the media was changed to DMEM/F12 + 10% FCS. The cells in flasks were shaken for 24hrs on day 8 at 275rpm at 37^0C. Between days 11 and 15, the near-confluent cells were plated at a density of 3×10^4 to 5×10^4 cells/ well in 12-well plates.

Capped cRNA was transcribed from the human brain glutamate transporter EAAT1 cDNAs by Dr. Kavanaugh's lab as described (Arriza et al., 1994). Transcripts were microinjected into Xenopus oocytes (50ng per oocyte) and uptake assay done 3-6 days later.

Transporter activity in C17.2 cells, HEK293T cells, primary astrocytes and oocytes: Transfected C17.2 and HEK293T cells in DMEM and primary astrocytes in DMEM/F12 containing 10% FCS were grown in a humid atmosphere of 5% CO2. Near-confluent cells were rinsed with a physiological buffer (138 mM NaCl, 11 mM D-glucose, 5.3 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 1.1 mM $CaCl_2$, 0.7 mM $MgSO_4$, 10 mM HEPES, pH 7.4) and allowed to preincubate at 37^0 C for 5 minutes. Uptake was initiated by replacing the pre-incubation buffer with buffer containing D-[^{3}H]aspartate and inhibitors. Following a 5 minute incubation, the media was removed by rapid suction and the cells rinsed three times with ice-cold buffer. The cells were dissolved in 0.4 N NaOH for 24 h and analyzed for radioactivity by LSC and protein by the BCA (Pierce) method. Transport rates were corrected for background, i.e., radiolabel accumulation at 4^0 C. Initial studies confirmed that uptake quantified in this manner was linear with time and protein levels and that uptake in untransfected C17.2 cells was indistinguishable from background.

Uptake was measured in control (uninjected) oocytes and in oocytes expressing EAAT1 during a 10-min incubation in ND-96 buffer (96mM NaCl, 2mM KCL, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES pH 7.4) containing D-[^{3}H]-aspartate and 10μM unlabeled D-aspartate. Uptake was terminated by three rapid washes in ice-cold buffer followed by lysis in 0.1% SDS and scintillation counting.

Curve fitting and statistical analysis: Kinetic analyses of the substrates in different

conditions were carried out using Kaleidagraph 3.6 (Synergy Software) by non-linear curve-fitting to Michelis-Menton equation. EC_{50} values for the dose-response curves were generated using a four-parameter Hill function using equation: y = a + b*c/(d^c+x^c), where a = y min, b = range of transition (y max – y min), c = slope, d = EC_{50}. When two groups were compared, a Student's *t* test was used to compare the values. Multiple groups were compared by ANOVA with post hoc analysis. A $p < 0.05$ was considered significant.

Chapter 3: Development and Validation of EAAT2 Binding-Site Pharmacophore Model

Introduction:

Glutamate is the major excitatory amino acid neurotransmitter in the mammalian CNS and is essential for normal functioning of the brain (Hollmann and Heinemann, 1994). A disruption in the control of glutamate homeostasis can lead to excitotoxicity (Olney, 1990; Doble, 1999). Five glutamate transporter subtypes, EAAT1-5, have been discovered that are responsible for maintaining optimal extracellular concentrations of glutamate (Danbolt, 2001; Robinson, 1999; Kanai and Hediger, 2003). However, the specific role that each individual EAAT subtype plays to maintain glutamate homeostasis is still being explored. One strategy to address this issue is through the development of subtype selective inhibitors.

The flexibility in the structures of both the ligands and the binding site of the target protein accounts for the ability of disparate ligands to bind to different subtypes of transporters that share a common substrate. Identifying chemical ligands that preferentially interact with particular EAAT subtype can provide insights into structural differences between transporters. Additionally, those substrates and inhibitors that exhibit little or no cross-reactivity with ionotropic or metabotropic glutamate receptors can be utilized as functional probes in physiological preparations.

EAAT PHARMACOLOGY:

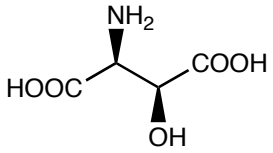
The constant search for potent and selective EAAT inhibitors has led to the discovery of several different chemical classes of compounds (Bridges et al., 1999; Dunlop and Butera, 2006; Mennini et al., 2003). The analogues can be divided between those that are transported across the membrane (i.e., alternative substrates) and non-transportable inhibitors that bind the transporter without being translocated. Based upon chemical similarity and competitive mechanism of inhibition, these molecules are hypothesized to interact with the same general binding site on the glutamate transporters. EAAT inhibitors can be broadly divided into several chemical classes on the basis of their differential effects on the glutamate transporters. Apart from making direct changes to the functional groups of the parent compounds, a wide variety of analogues have been generated via modifications of the carbon backbone of the parent molecule. These alterations could be achieved by either the introduction of certain chemical groups to the carbon backbone or restricting the positions that the functional groups can assume in three dimensional (3D) space by introducing a ring system. The successful implementation of these strategies has lead to the development of an array of compounds that are not only better inhibitors in terms of potency but also have properties that may be used to probe the unique characteristics within the binding sites of different EAAT subtypes.

β-Substituted aspartate derivatives: One of the earliest competitive inhibitors identified (Balcar and Johnston, 1972; Robinson et al., 1993), DL-β-*threo*-hydroxyaspartate (β-THA) (Figure 3.1) has been shown to bind all EAAT subtypes with substrate-like activity at EAAT1-4 (Lebrun et al., 1997) and non-transportable inhibitor-like properties at

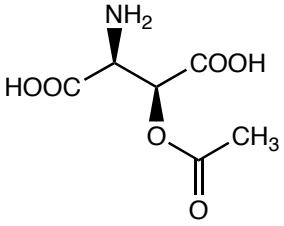
EAAT5 (Arriza et al., 1997). Initial studies have characterized a series of derivatives in which the β-hydroxyl group was esterified to yield *threo*-β-acetoxy (TAcOAsp), propionyloxy (TPnOAsp), benzoyloxy (TBzOAsp), (1-naphthoyl)oxy (T1NpOAsp) and (2-naphthoyl)oxy (T2NpOAsp) THA analogues (Lebrun et al., 1997). Electrophysiologically, TAcOAsp and TPnOAsp were found to elicit substrate-induced currents in EAAT1-expressing oocytes with K_m values of 40μM and 64μM respectively. On the other hand, TBzOAsp and T1NpOAsp were shown to be non-transportable inhibitor with the K_i values of about 17μM and 52μM respectively at blocking L-glutamate-induced currents in oocytes expressing EAAT1.

More recently, further modifications of β-THA have led to development of some of the most potent, non-tranportable inhibitors available for the glutamate transporters (Shimamoto et al., 1998; Shimamoto et al., 2004). The most prominent analogues of this class are L-β-*threo*-benzyloxy aspartate (L-β-TBOA) (Shimamoto et al., 1998) and (2*S*,3*S*)-3-(3-[4-(trifluoromethyl)benzoylamino]benzyloxy)aspartate (TFB-TBOA) (Shimamoto et al., 2004). L-β-TBOA was found to be potent inhibitor of both EAAT1 and 2 expressed in COS-1 cells and oocytes. The K_i values for this non-transportable inhibitor were found to be about 0.12μM and 9μM in oocytes expressing EAAT2 and EAAT1 respectively. Moreover, L-β-TBOA did not inhibit radiolabel binding at ionotropic and metabotropic glutamate receptors. Recently, this group has synthesized another very potent β-THA analogue, TFB-TBOA that exhibited IC_{50} values for EAAT1-3 in the nanomolar range when tested for the inhibition of L-[^{14}C]-glutamate uptake in COS-1 cells expressing individual EAAT subtypes (Shimamoto et al., 2004). It showed

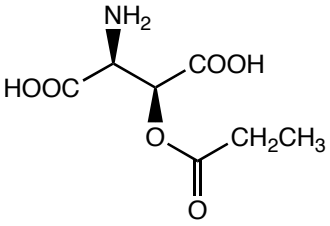
Figure 3.1. β-Substituted aspartate derivatives.



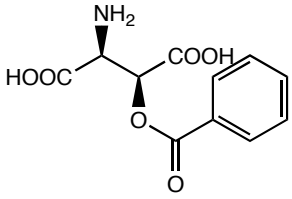
L-β -THA



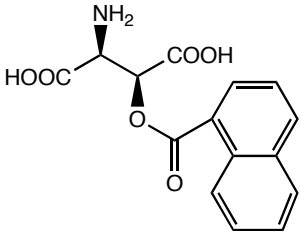
L-β -TAcOAsp



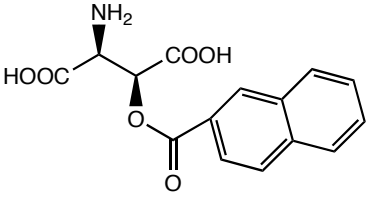
L-β -TPnOAsp



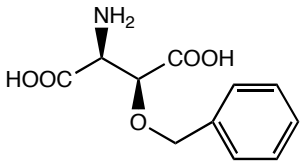
L-β -TBzOAsp



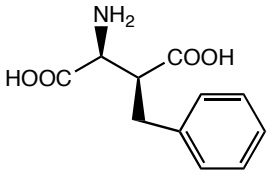
L-β -T1NpOAsp



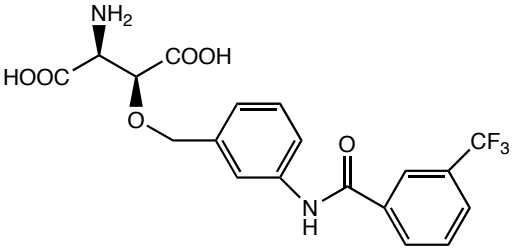
L-β -T2NpOAsp



L-β -TBOA



L-β -TBA



TFB-TBOA

an ~15-fold preference for EAAT1 and EAAT2 over EAAT3. In the same study, TFB-TBOA elicited no cross-reactivity with ionotropic glutamate receptors as determined by the radioligand binding assay on synaptic membranes or with metabotropic glutamate receptors when tested in transfected CHO cells.

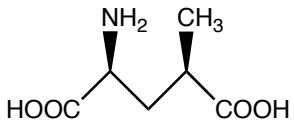
Based on the properties of L-β-TBOA, the Esslinger group replaced the benzyloxy group with the benzyl group yielding L-β-*threo*-benzylaspartate (L-β-TBA). Significantly, this analogue is among the first compounds to show ~10-fold preference for EAAT3 over EAAT1 and EAAT2 when tested in C17.2 cells and in oocytes (Esslinger et al., 2005). L-β-TBA was found to be a competitive non-tranportable inhibitor with K_i values of 10.2μM, 11.4μM and 1.2μM at EAAT1,2 and 3 respectively in oocytes. With both the β-benzyl- and the β-benzyloxy- analogues, the *threo*- stereoisomer was found to be significantly more potent than the *erythro*- form (Esslinger et al., 2005), (Shimamoto et al., 2000). Taken together, these results suggest that the addition of bulky substituents to the β-carbon, convert a substrate to a non-transportable inhibitor. It has been suggested that these groups likely exhibit greater inhibitory activity because of their ability to participate in the hydrophobic interactions within the binding site of the glutamate transporters (Esslinger et al., 2005; Dunlop et al., 2005).

3- and 4-Substituted glutamate derivatives: Similar to the substituted aspartate analogues, additions to the carbon backbone of glutamate have produced a series of important analogues (Figure 3.2). Methyl substitution at C-3 of glutamate converts it to a molecule that shows differential effects at EAAT subtypes (Vandenberg et al., 1997;

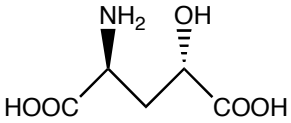
Eliasof et al., 2001). The studies on EAAT1-4 expressed in oocytes and MDCK cells revealed that *threo*-3-methylglutamate (T3MG) is a potent and competitive blocker of glutamate transport by EAAT2 and EAAT4, but only a weak inhibitor of EAAT1 and EAAT3 (Eliasof et al., 2001). Whereas, T3MG appears to be a non-transportable inhibitor of EAAT2 (Vandenberg et al., 1997; Eliasof et al., 2001), it interacts with the EAAT4 with substrate-like activity (Eliasof et al., 2001). The IC_{50} values for EAAT2 and EAAT4 were reported to be 90μM and 109μM respectively in oocytes (Eliasof et al., 2001). T3MG also elicited weak NMDA agonist activity. The *erythro*-3-methylglutamate (E3MG) was shown to be inactive at EAAT1 and 2 when tested in oocytes (Vandenberg et al., 1997).

Additionally, a number of studies have been done to characterize the effects of substitutions at C-4 of glutamate. Both, *threo*- and *erythro*- isomers of 4-hydroxyglutamate (4HG) have been demonstrated to retain the substrate activity at EAAT1 and EAAT2 when tested in oocytes (Vandenberg et al., 1997). However, the L-*threo*- isomer of 4HG was found to be much more potent (K_m of 61μM and 48μM at EAAT1 and 2 respectively) than the *erythro*- form (K_m ~1mM at EAAT1 and EAAT2). 4-Methyl substitution of glutamate also yields molecules with differential activities (Vandenberg et al., 1997; Alaux et al., 2005). When studied in oocytes (2S, 4R)-4-Methylglutamate ((2S, 4R)-4MG) was found to exhibit substrate properties at EAAT1 with K_m of 54μM and I_{max} of 80% relative to glutamate. However, it acts as a non-transportable inhibitor of EAAT2 and EAAT3 (Alaux et al., 2005). Interestingly,

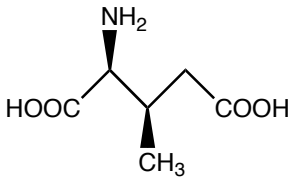
Figure 3.2. 3- and 4-Substituted glutamate derivatives.



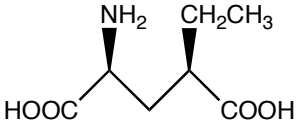
2S,4R-4-Methyl glutamate



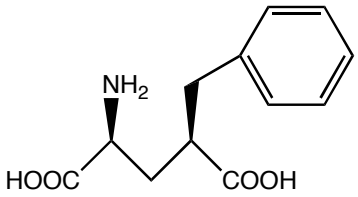
L-*threo*-4-Hydroxy glutamate



L-*threo*-3-Methyl glutamate



2S,4R-4-Ethylglutamate

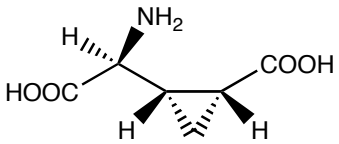


2S,4R-4-Benzylglutamate

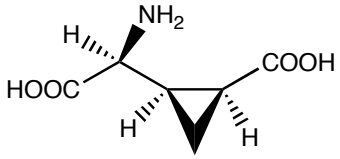
extending the 4-substitution of glutamate from methyl to higher alkyl or benzyl group retains inhibitory activity, but converts them to non-transportable inhibitors at EAAT1, 2 and 3 as characterized in FLIPR-based membrane potential (FMP) assay (Alaux et al., 2005). In terms of cross-reactivity, 2S,4R-4MG shows potent agonist ability at kainate receptors and inhibits ^{3}H-kainate binding to membranes prepared from CNS tissue (Gu et al., 1995; Brauner-Osborne et al., 1997). While it was shown to inhibit the binding of ^{3}H-CPP to NMDA receptors, it exhibited no activity at AMPA receptors.

2-(Carboxycyclopropyl)- and 2-(carboxybutyl)- glycine analogues: These constrained analogues lock the β- and γ- positions of glutamate by introducing a ring into the structure, thereby limiting the number of conformations it can attain (Figure 3.3). The availability of these probes has proven to be beneficial for developing pharmacophores for glutamate transporters. Among the L-2-(2-carboxycyclopropyl)glycines (CCGs), the analogues: L-CCG-III (2S,3S,4R isomer) and L-CCG-IV (2S,3R,4S isomer), have proven to be particularly important. L-CCG-III has been reported to be the most potent analogue in terms of its activity at glutamate carriers. It was demonstrated to be a potent competitive inhibitor of L-[^{3}H]-glutamate uptake in COS-1 cells expressing EAAT2 with IC_{50} values of 0.29μM (Yamashita et al., 1995). When studied for its activity at inhibiting L-[^{14}C]-glutamate uptake in COS-1 cells, the K_i values for L-CCG-III were reported to be 7.5μM and 2.5μM for EAAT1 and EAAT2 respectively (Shimamoto et al., 1998). In another study, L-CCG-III was reported to be effective at blocking L-[^{3}H]-glutamate uptake in C6 glioma cells, which expresses EAAT3 but not EAAT1 or EAAT2 (Palos et al., 1996), and EAAT3 expressing oocytes with reported K_i values of 10μM and 13μM

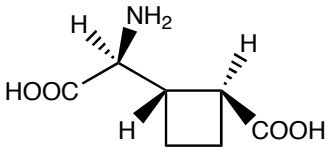
Figure 3.3. 2-(Carboxycyclopropyl)- and 2-(carboxybutyl)- glycine analogues.



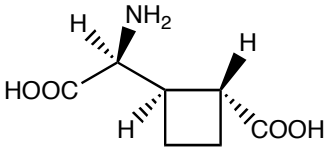
L-CCG-III



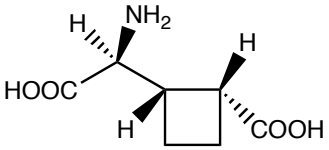
L-CCG-IV



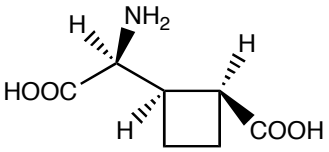
L-CBG-I



L-CBG-II



L-CBG-III



L-CBG-IV

respectively (Dowd et al., 1996). Although not quiet as potent, L-CCG-IV was shown to inhibit the EAAT2-mediated L-[^{3}H]-glutamate uptake in COS-1 cells with an IC_{50} value of 1.1μM (Yamashita et al., 1995). However, when another study tested L-CCG-IV in COS-1 cells, it exhibited competitive inhibitor activity of L-[^{14}C]-glutamate uptake with IC_{50} values of 900μM and 673μM for EAAT1 and EAAT2 respectively (Shimamoto et al., 1998). Similarly, L-CCG-IV was reported to competitively block the L-[^{3}H]-glutamate uptake in oocytes with K_i value of 171μM at EAAT3 (Dowd et al., 1996). Both L-CCG-III and L-CCG-IV have been shown to bind ionotropic glutamate receptors. While L-CCG-III was shown to inhibit the binding of [^{3}H]-kainate in forebrain synaptic membranes, it exhibited no activity at NMDA or AMPA receptors (Kawai et al., 1992). L-CCG-IV was reported to have potent depolarizing ability mediated primarily through interaction with NMDA receptors in isolated rat spinal cord (Shinozaki et al., 1989). Additionally, L-CCG-IV also inhibited the binding of [^{3}H]-CPP, [^{3}H]-kainate and [^{3}H]-AMPA to synaptic membranes of the rat brain (Kawai et al., 1992).

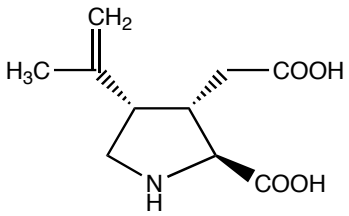
Four stereoisomers of L-2-(2-carboxycyclobutyl)glycine (L-CBG): L-CBG-I (2S,1´S,2´S), L-CBG-II (2S,1´R,2´R), L-CBG-III (2S,1´S,2´R), and L-CBG-IV (2S,1´R,2´S), have been characterized in HEK293 cells transfected with EAAT1, 2 and 3 by FLIPR membrane potential (FMP) assay (Faure et al., 2006). L-CBG-I and L-CBG-III appeared to be weak substrate and non-transportable inhibitors respectively at EAAT1, 2 and 3. L-CBG-II showed differential activities at these transporters. While shown to act as a substrate at EAAT1 (K_m = 96μM), L-CBG-II potently inhibited the uptake of L-glutamate by EAAT2 and EAAT3 with K_i values of 22μM and 49μM respectively. In the

same study, L-CBG-IV was found to be a weak inhibitor at EAAT1 (K_i 200μM), but a moderately potent inhibitor at EAAT2 and EAAT3 (K_i 6.6μM and 10 μM).

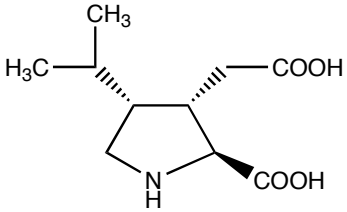
Pyrrolidine dicarboxylate (PDC) derivatives: This class represents compounds that are constrained analogues of aspartate or glutamate molecules (Figure 3.4). Introduction of pyrrolidine heterocycle limits the number of conformations that the compounds can assume, and has proven to be important in the development of transporter pharmacophores.

Initial studies have recognized kainate and dihydrokainate to be non-transportable inhibitors of glutamate transport (Johnston et al., 1979; Bridges et al., 1999). Whereas, kainate is a proven agonist at KA receptors, its modification to DHK was shown to markedly reduce binding to KA and AMPA receptors while enhancing the uptake inhibitor activity. The isopropyl side chain of DHK appears to be an important determinant of its activity. 2-Carboxy-3-pyrrolidineacetate, in which the isopropyl group is absent, is a poor inhibitor of glutamate transport with enhanced binding capacity to KA, AMPA and NMDA receptors (Sonnenberg et al., 1996). Later, DHK was shown to be an extremely valuable ligand, as it selectively inhibits EAAT2, the most prevalent glutamate transporter in the brain (Arriza et al., 1997; Arriza et al., 1994; Fairman, 1995; Shimamoto et al., 1998; Vandenberg et al., 1997). 2,4-Pyrrolidine dicarboxylates represent constrained glutamate analogues. L-*trans*-2,4-PDC (2,4-PDC) has been shown to be the most potent isomer with respect to the inhibition of glutamate uptake (Garlin et al., 1995; Koch et al., 1999; Dowd et al., 1996; Griffiths et al., 1994) with reported K_m

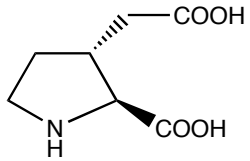
Figure 3.4. Pyrrolidine dicarboxylate (PDC) derivatives.



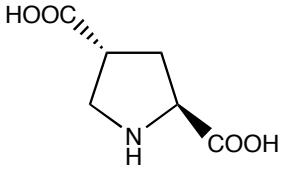
Kainic acid



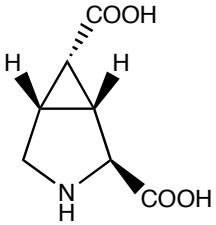
DHK



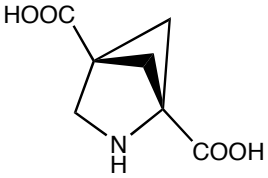
CPAA (2-carboxy-3-pyrrolidineacetic acid)



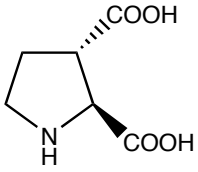
L-*trans*-2,4-PDC



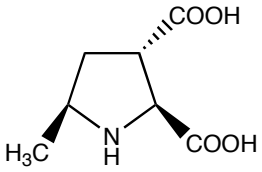
L-*anti-endo*-3,4-MPDC



2,4-MPDC



L-*trans*-2,3-PDC



***cis*-5-Me-L-*trans*-2,3-PDC**

values comparable to that of glutamate at EAAT1 (28μM), EAAT2 (7μM), EAAT3 (27μM) and EAAT4 (2.6μM) in oocytes (Arriza et al., 1994; Fairman, 1995). While it acts as a substrate at EAAT1-4, L-2,4-PDC was shown to be non-transportable inhibitor of EAAT5 when expressed in oocytes (Arriza et al., 1997). As regards cross-reactivity with glutamate receptors, L-2,4-PDC did not show binding to ionotropic EAA receptors in radioligand binding assay (Bridges et al., 1991). However, some cross-reactivity with metabotropic receptors has been reported in cultured astrocytes (Miller et al., 1994). More recent studies have evaluated the effects of modifications on L-2,4-PDC. Addition of a methyl group to form 2S,4R-4-methyl-PDC was shown to convert a substrate into a competitive, non-transportable inhibitor of D-[^{3}H]-aspartate uptake when tested in rat forebrain synaptosomes (Esslinger et al., 2002). Methyl group addition can be assumed to invoke additional interaction with the binding site or confer steric hindrance within the molecule.

The 2,3-pyrrolidine dicarboxylates contain an embedded aspartate template. Unlike L-2,4-PDC, L-*trans*-2,3-PDC (L-2,3-PDC) has been shown to exhibit non-transportable inhibitor characteristics with a K_i of 23μM against D-[^{3}H]-aspartate uptake in rat forebrain synaptosomes (Willis et al., 1996). Significantly, while the activity of L-2,3-PDC was confirmed in oocytes (K_i = 10μM), it was reported to have little or no activity as an inhibitor at EAAT1 or EAAT3 (Bridges et al., 1999). Furthermore, the addition of a methyl susbstituent at the 5-position of L-*trans*-2,3-PDC has been shown to produce additional changes in activity at EAAT2. Whereas, the *cis*-5-methyl derivative retains the inhibitor activity without compromising the potency, the *trans*- addition abolishes its

activity (Willis et al., 1997). In terms of cross-reactivity, L-2,3-PDC has been shown to be an excitotoxin and is a potent NMDA receptor agonist. The radioligand binding assays have shown L-2,3-PDC to bind NMDA, KA and AMPA receptors in rat brain (Willis et al., 1996).

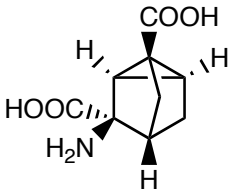
The PDCs have further been constrained by introducing a methano-bridge between α- and γ- carbons or β- and γ- carbons to yield 2 important analogues: 2,4-methanopyrrolidine-2,4-dicarboxylate (2,4-MPDC) and L-*anti*-*endo*-3,4-methano-pyrrolidine-3,4-dicarboxylate (L-*anti*-*endo*-3,4-MPDC). The linking of the PDC backbone reduces the number of conformations that the molecule can assume, thus, making it possible to identify the "preferred" conformer at the binding site. Both 2,4-MPDC and L-*anti*-*endo*-3,4-MPDC were shown to be potent competitive inhibitors of D-[^{3}H]-aspartate uptake into rat forebrain synaptosomes (Esslinger et al., 1998). Interestingly, while L-*anti*-*endo*-3,4-MPDC was identified as a potent non-transportable competitive inhibitor, 2,4-MPDC exhibited excellent substrate characteristics with slightly lower potency when tested in oocytes expressing EAAT2.

Taken together, the above results can help highlight key interactions within the binding domains of individual glutamate transporter subtypes. Pharmacologically, more EAAT2-preferring ligands have been identified than those exhibiting selectivity for other EAATs. DHK and L-2,3-PDC were among the first compounds to elicit highly selective inhibitor activity at EAAT2 (Arriza et al., 1994; Arriza et al., 1997; Fairman, 1995; Shimamoto et al., 1998; Vandenberg et al., 1997). Although not quite as selective, compounds like L-

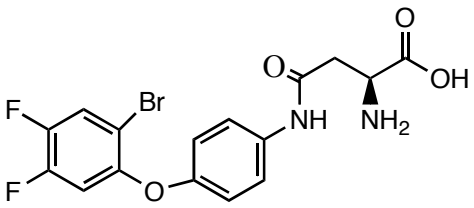
anti-endo-MPDC (Esslinger et al., 1998), L-CCG-IV (Shimamoto et al., 1998; Yamashita et al., 1995), S-2-amino-3-(3-hydroxy-1,2,5-thiadiazol-4-yl) propionic acid ((S)-TDPA) (Brauner-Osborne et al., 2000), WAY213613 (Dunlop et al., 2005) and WAY855 (Dunlop et al., 2003) show preference for binding EAAT2 over other EAATs (Figure 3.5). In the instance of EAAT1 and EAAT3-5, only subtle distinguishing differences have emerged. EAAT1 show substrate-like activity for compounds like L-SOS (Arriza et al., 1994; Vandenberg et al., 1998b), (2S,4R)-4MG (Vandenberg et al., 1997) and LCBG-II (L-2-(2-carboxycyclobutyl) glycine isomer) (Faure et al., 2006) and is more potently inhibited by 1-hydroxy-1,2,3-triazol-5yl propionate (Stensbol et al., 2002). L-Cysteine and L-aspartate-β-hydroxamate has been shown to act at EAATs and exhibit preferential activity for EAAT3 compared to EAAT1 or EAAT2 (Zerangue and Kavanaugh, 1996b; Roberts and Watkins, 1975). While exhibiting substrate properties at other subtypes, L-2,4-PDC and THA have been shown to be non-transportable inhibitors at EAAT5 (Arriza et al., 1997).

Insight into the requirements necessary for a molecule to act as an inhibitor can be gained and visualized from comparing the commonalities and differences among the identified ligands by generating a pharmacophore model (Mason et al., 2001). A pharmacophore is the spatial mutual orientation of atom or groups of atoms assumed to be recognized by and interact with the particular binding site. Thus, a pharmacophore specifies the spatial relationships between the groups in 3D space. Pre-shaping the ligands to the geometry of the binding site for mutual molecular recognition in order to minimize the loss of conformational

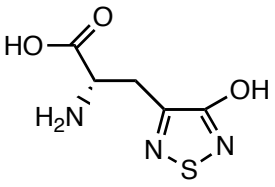
Figure 3.5. Other EAAT ligands.



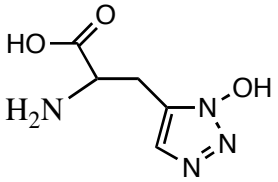
WAY-855



WAY-213613



(S)-TDPA



1-Hydroxy-1,2,3-triazol-5-yl-propionate

entropy upon binding and improving ligand protein interactions to obtain a favorable (negative) enthalpy change, are important factors that can lead to the improved binding affinity as well as reduce cross-reactivity (Velazquez-Campoy et al., 2001; D'Aquino et al., 2000).

In this study, we have built an EAAT2-specific binding site pharmacophore model by mapping the functional groups on the specific training set ligands and calculating the structural similarities in 3D space (Dean and Perkins, 1998; Perkins and Dean, 1993; Martin, 1998). This model predicts distinct regions that might influence the potency and selectivity of the EAAT2 ligands, including: 1) a highly conserved positioning of the two carboxylate Cs and the amino N, 2) a nearby region that can accommodate selective modifications (e.g., cyclopropyl ring, CH_3 groups, and O atoms), and 3) the region occupied by the benzyl ring of L-TBOA. Additionally, we have incorporated a novel EAAT3-preferring inhibitor L-β-*threo*-benzyl-aspartate (L-β-TBA) into our model in an attempt to identify certain plausible differences between the interactions within the EAAT2 and EAAT3 binding sites. Comparison of the superpositioned L-β-TBOA and L-β-TBA in our model suggests that the selective activity at EAAT binding sites may reside in the location and relative orientation of an aromatic ring moiety, as well as the composition of the linking atoms attaching the aromatic ring to the aspartyl backbone of these ligands.

Chapter 3: Results

Four EAAT2 inhibitor training set ligands, i.e. L-*anti*-*endo*-3,4-methanopyrrolidine-3,4-dicarboxylate (MPDC), *cis*-5-methyl-L-*trans*-2,3-PDC (PDC; 2S,3R,4S)-2-(carboxy-cyclopropyl)glycine (CCG-IV) , and β-*threo*-benzyloxy-aspartate (L-β-TBOA) were selected to build an EAAT2 binding-site pharmacophore model. Each of the significant EAAT2 inhibitor classes (Bridges et al., 1999) was carefully considered for inclusion in the training set (Table 3.1). The major criteria were high potency, structural diversity amongst common moieties, and relatively high selectivity. Importantly, only potent inhibitors with little or no substrate activity were selected to afford a pharmacophore model that may be used to define the key structural requirements for a compound to bind to the transporter and inhibit its activity.

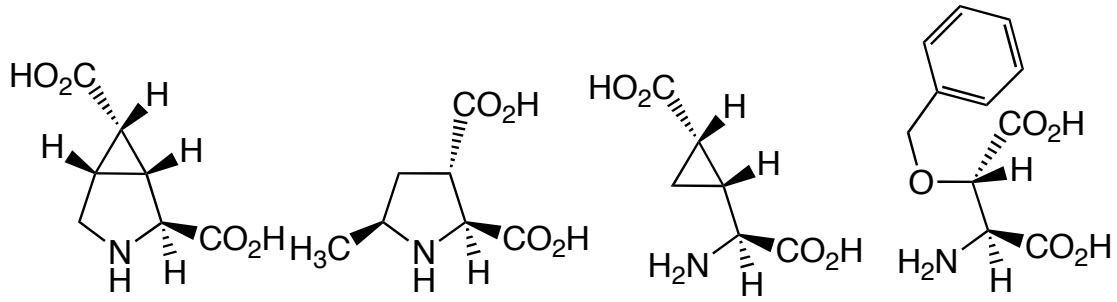
Most molecules can adapt more than one conformation of nearly equal energy by rotation around single bonds. These molecular geometries correspond to the global and, in most cases, various local minima on the multidimensional molecular energy surface (also called potential energy surface). The conformation with the global minimum energy seldom binds to the target protein (Nicklaus et al., 1995; Perola and Charifson, 2004). Moreover, the "preferred" conformation depends on the interactions of the molecule with its environment. In structure-based drug design, the so-called bioactive conformer (the preferred conformation in the receptor-bound state) of potential drug molecules is of special interest. The major aim of conformational analysis is to identify the preferred conformations of a molecule under specific conditions. Therefore, conformational search techniques (i. e., methods that locate the global and local energy minima of a structure) play a crucial role in conformational analysis. To ensure that the bioactive conformation

is included, a wide collection of conformations was used. Conformational space, i.e., the total number of possible conformations a molecule can assume, was rigorously searched using two stochastic methods (Saunders, 1987; Chang et al., 1989) e.g., random search (Tripos) and AESOP (Masek, 1998) rather than a systematic search that examines every possible configuration that a molecule can assume (Smellie et al., 1995b; Smellie et al., 1995a). Separate conformational molecular databases for each training set ligand were formed that contained all the conformers from all the searches with the duplicates and nearly identical rotamers removed. The energy range and number of conformers for these four ligands are shown in Table 3.2.

All conformers of all the ligands were compared to each other (combinatorial conformer approach) based on three distances and three angles. The requirement of a minimum of three points to overlay in three-dimensional (3D) space (Marshall, 1995) was met by three selected points of interest (N, C1 and C2) that were shared among the ligands (Figure 3.6). The six measures included three distances and three angles between these points (Figure 3.1 Table). In addition to the distal carboxylate carbon of all the ligands, the electron-rich benzyloxy 'O' of L-β-TBOA and the 'centroid' of LCCG-IV were considered as point C2 to generate four plausible pharmacophore models.

While the proximal carboxylate carbon (C1) and amino nitrogen (N) were common in all models the distal carboxylate carbon (C2) (Model A) was replaced by either the benzyloxy 'O' of TBOA (Model B and D) or cyclopropyl 'centroid' of CCG-IV (Model C and D). The comparison of the ligands was scored (Dean and Perkins, 1998) for

Table 3.1. Criteria for the selection of training set.



	L-*anti-endo*-3,4-MPDC		*cis*-5-*Me*-L-*trans*-2,3-PDC		L-CCG-1V		L-β-TBOA	
	K_i (μM)	I_{max} (% Glu)	K_i (μM)	I_{max} (% Glu)	K_i (μM)	I_{max} (% Glu)	K_i (μM)	I_{max} (% Glu)
EAAT1	30	21	630	0	900*	94	9	0
EAAT2	***1.6***	***0***	***11***	***0***	***673****	***20***	***0.12***	***0***
EAAT3	45	35	125	0	-	-	-	-
EAAT4	3.8	58	>3mM	-	-	-	-	-

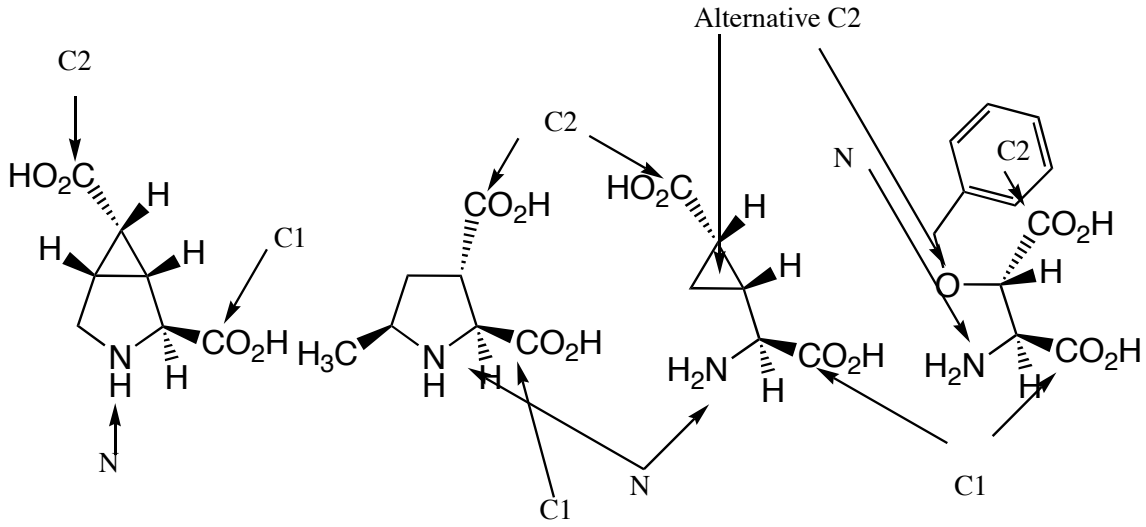
Inhibitory activity of ligands selected in the training set for the development of EAAT2 specific pharmacophore model. * IC_{50}.

Table 3.2. Number of unique conformers and energy range for each of the ligands in the training set.

	L-*anti-endo*-3,4-MPDC	*cis*-5-*Me*-L-*trans*-2,3-PDC	L-CCG-IV	L-β-TBOA
# Conformers	100	107	440	208
Energy Range (kcal/mol)	107.158-157.484	10.006-91.617	103.637-189.635	1.635-1116.144

Conformational space was searched using two stochastic techniques: random search and AESOP. These unique conformers were obtained after the removal of duplicates.

Figure 3.6. Training set with points-of-interest labeled.



L-*anti-endo*-3,4-MPDC *cis*-5-*Me*-2,3-PDC L-CCG-IV L-β-TBOA

DISTANCE			ANGLE		
N-C1	N-C2	C1-C2	N-C1-C2	N-C2-C1	C1-N-C2

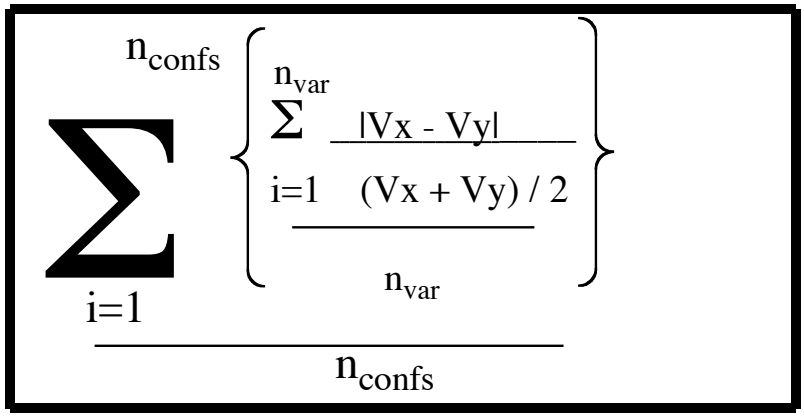
The points-of-interest: amino nitrogen (N), proximal (α) carboxylate carbon (C1) and distal carboxylate carbon (C2), were selected that were shared among all the ligands in the training set. For each comparison group, six distinct measures (three distances and three angles) were defined between each ligand conformer for the assessment of molecular similarity. Additionally, the ether oxygen of L-β-TBOA and the cyclopropyl centroid of L-CCG-IV were substituted for point C2 to consider alternative relative alignments.

relative structural similarity in 3D space using Equation 1 (Figure 3.7). The selected ligand conformer set was brought together (multifit) with a 1 cal spring constant. The final superposition models (Figures 3.8-3.11) were selected on the basis of their scoring function values (Charts in Figures 3.8-3.11) and visual inspection using CrystalEyes viewers. The best superposition models exhibited low scoring function values as expected.

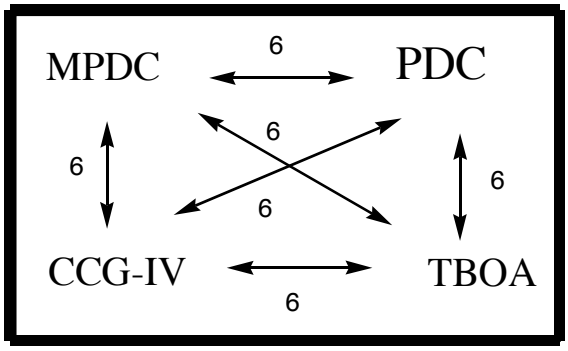
Based upon the stringent alignment of the carboxylate groups, the amino moieties and the carbon backbone, model A was selected (Figure 3.8). The other models, which were based upon the superpositioning of the ether-O of L-β-TBOA at the C2 carboxylate position (Model B), the centroid of the cyclopropyl ring of L-CCG-IV at the C2 carboxylate position (Model C), or both (Model D), were not considered further because each showed inappropriate positioning of the carbon backbone and/or relevant side chains. For instance, in models C and D, the carbon backbone markedly deviated outside the bounds defined by the other analogues. Figure 3.12 shows model A with critical distances and angles between the various functional groups listed in an inset along with the graphical representation in the x-y plane. The generated model suggests some key interactions that may contribute to their activity as well as selectivity of this group of ligands. These include the almost planar arrangement of the α-C, β-C, amino-N and distal carboxylate-C atoms. The identical positioning of the two carboxylate carbon atoms among the inhibitors suggests a critical electrostatic interactions between these groups and corresponding residues in the binding site and / or cotransported ions.

Figure 3.7.

Equation 1.

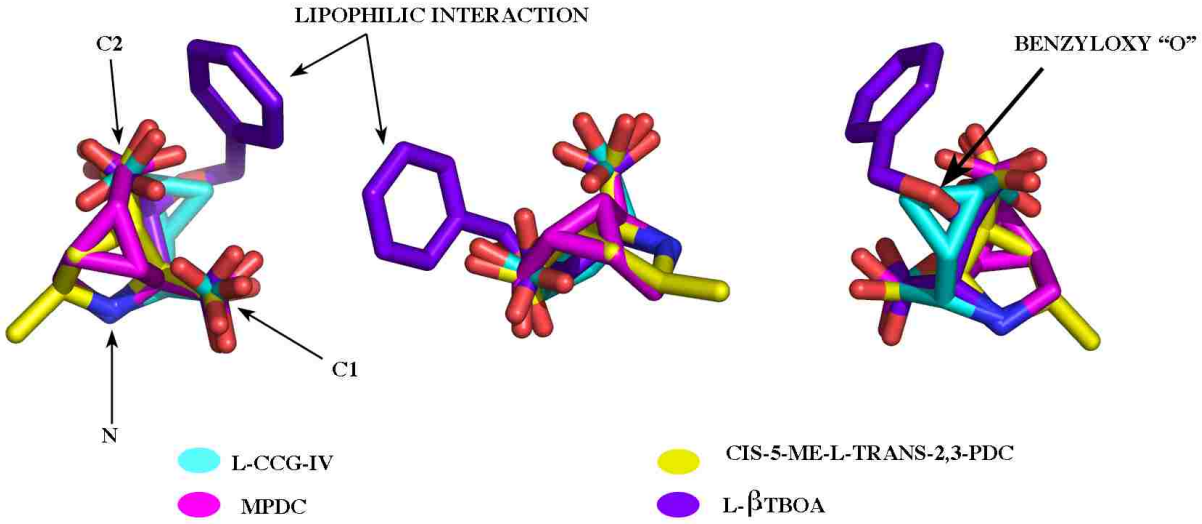


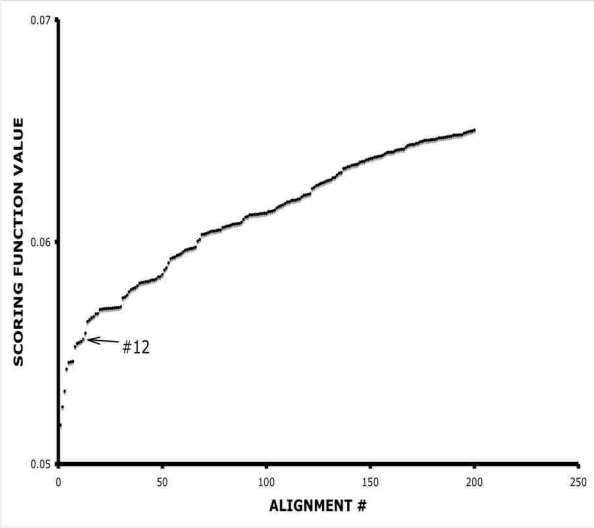
B.



Equation 1: V are 6 variable measures (3 distances and 3 angle) as compared for ligand x versus ligand y. n_{var} = 6 (measures); n_{confs} = 6 per comparison group regime (B). The relative difference comparison measures between the conformers in a group (double headed arrows) included three distances and three angles.

Figure 3.8. EAAT2 binding-site pharmacophore model A.

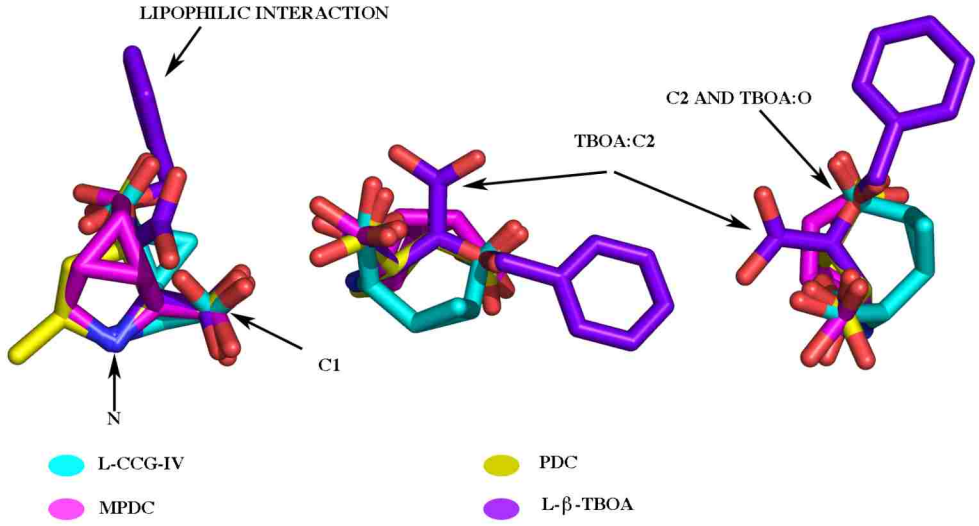


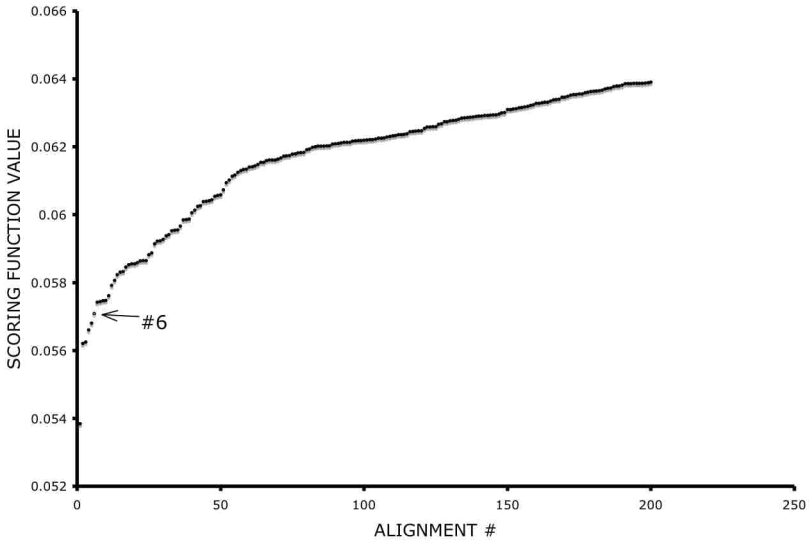


The chart shows the distribution of different alignment groups based on the scoring function value. Only top 200 alignment groups are shown. The alignment group # 12 was chosen based on the visual inspection using CrystalEyes viewers. The points-of-interest are indicated in the color-coded images. Three views of the same model are shown.

Figure 3.9. EAAT2 binding-site pharmacophore model B.

	DISTANCE			ANGLE		
CCG PDC MPDC	N-C1	N-C2	C1-C2	N-C1-C2	N-C2-C1	C1-N-C2
TBOA	N-C1	N-O	C1-O	N-C1-O	N-O-C1	C1-N-O

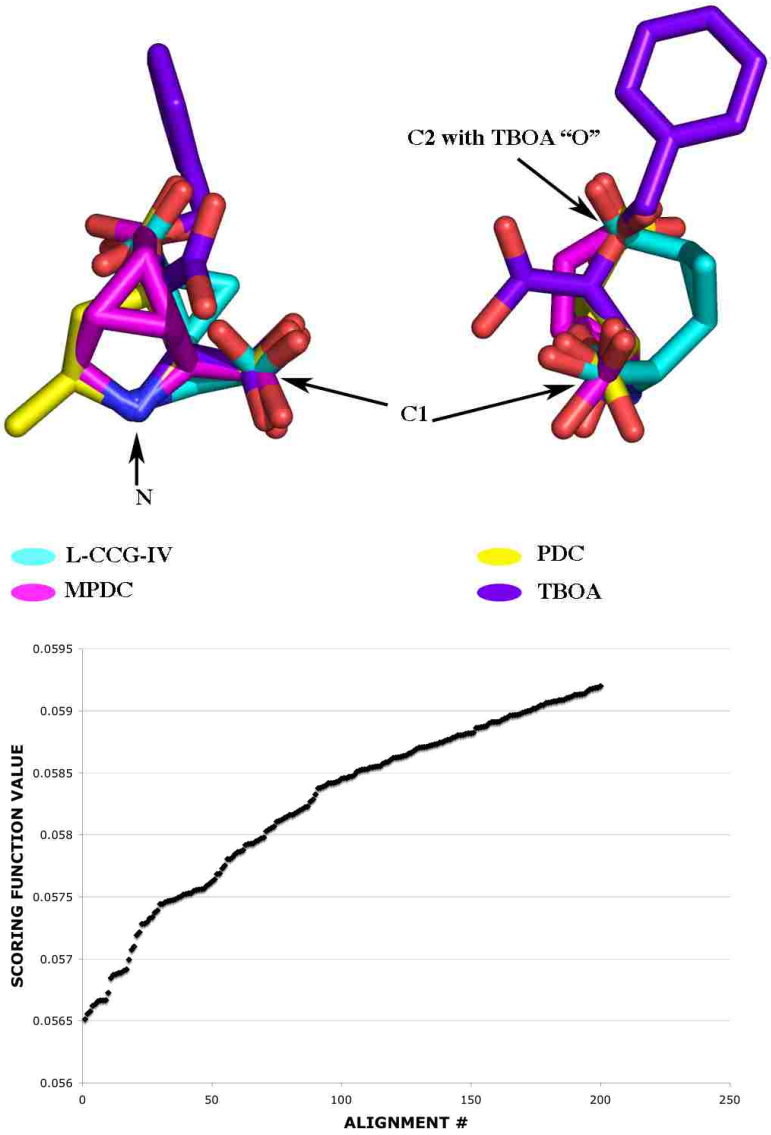




In model B, the distal carboxylate carbon of L-β-TBOA is substituted with the benzyloxy oxygen as point C2 . The chart shows top 200 alignment groups. The alignment group # 6 was chosen on the basis of the assessment done using CrystalEyes viewers. The points-of-interest are indicated in the color-coded images. Three views of the same model are shown. The table shows the measures used for the alignment of the training set ligands.

Figure 3.10. EAAT2 binding-site pharmacophore model C.

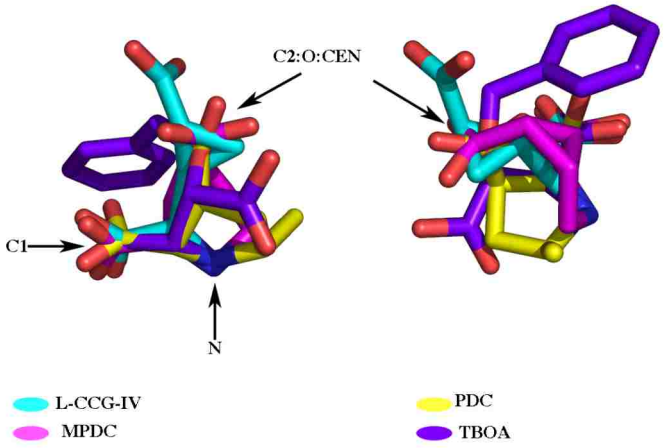
	DISTANCE			ANGLE		
PDC MPDC TBOA	N-C1	N-C2	C1-C2	N-C1-C2	N-C2-C1	C1-N-C2
CCG	N-C1	N-CEN	C1-CEN	N-C1-CEN	N-CEN-C1	C1-N-CEN

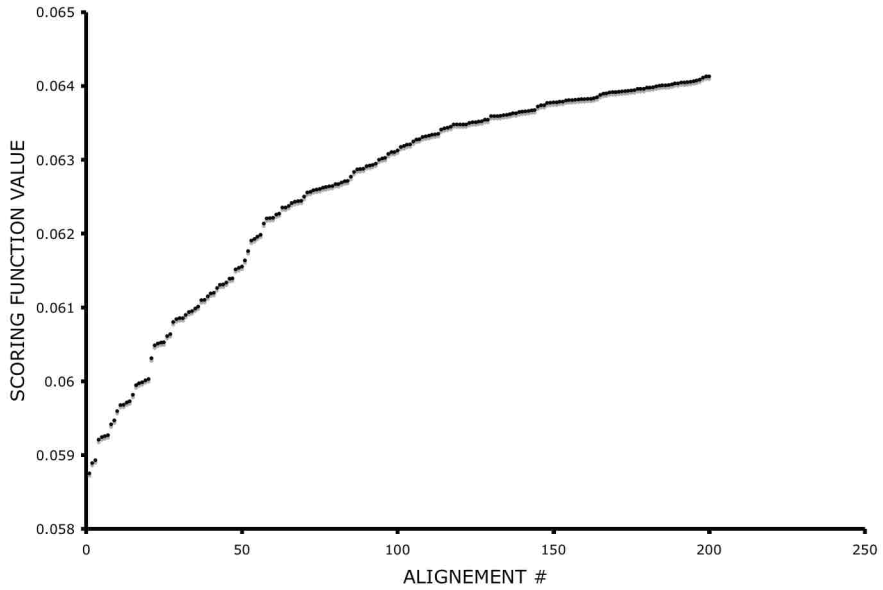


In model C, the distal carboxylate carbon of L-CCG-IV is replaced by the cyclopropyl centroid as point C2 (Table). The chart shows the distribution of different alignment groups based on the scoring function values. The points-of-interest are indicated in the color-coded images. Two views of the same model are shown. The table shows the measures used for the alignment of the training set ligands.

Figure 3.11. EAAT2 binding-site pharmacophore model D.

	DISTANCE			ANGLE		
PDC MPDC	N-C1	N-C2	C1-C2	N-C1-C2	N-C2-C1	C1-N-C2
CCG	N-C1	N-CEN	C1-CEN	N-C1-CEN	N-CEN-C1	C1-N-CEN
TBOA	N-C1	N-O	C1-O	N-C1-O	N-O-C1	C1-N-O

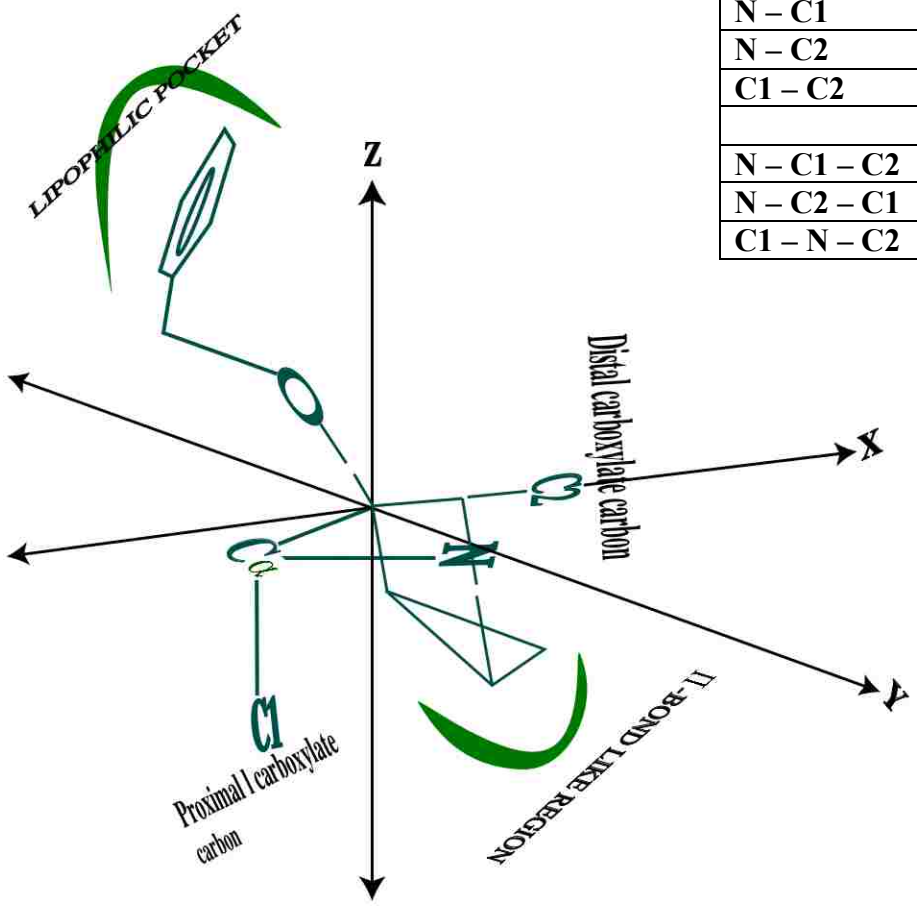




In model D, the distal carboxylate carbons of L-CCG-IV and L-β-TBOA are replaced by the cyclopropyl centroid and the benzyloxy oxygen as point C2 (Table). The chart shows the distribution of different alignment groups based on the scoring function values. The points-of-interest are indicated in the color-coded images. Two views of the same model are shown. The table shows the measures used for the alignment of the training set ligands.

Figure 3.12. Graphical representation of EAAT2 binding-site pharmacophore model A.

	Distance (Å)
N – C1	2.5 ± 0.1
N – C2	3.4 ± 0.2
C1 – C2	3.4 ± 0.2
	Angle
N – C1 – C2	66 ± 0.4
N – C2 – C1	43 ± 1.3
C1 – N – C2	71 ± 1.3



This representation depicts the averaged position of structural features. The averaged specific angle and distance measurements are reported in the table.

Secondly, the possibility of lipophilic interaction between the phenyl ring and the hydrophobic residues within the binding site. Additionally, positioning of the cyclopropyl ring of L-*anti*-*endo*-3,4-MPDC suggest a possible Π bond-like interaction with the protein.

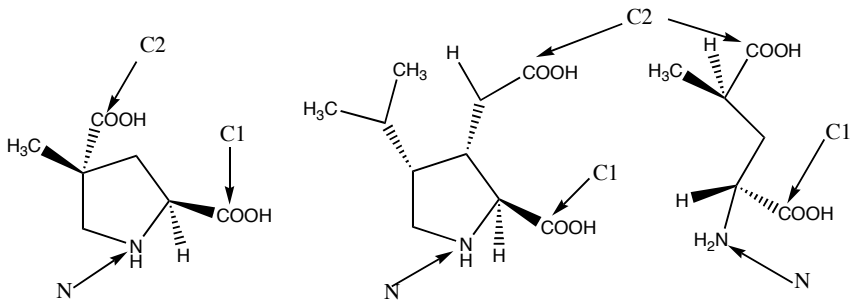
Pharmacophore Validation: It is important that the generated pharmacophore be able to predict the potential activity of an unknown ligand. Therefore, to check the quality of the model, it was validated with three test ligands using a "leave-one-out protocol" (Marshall, 1995). Three potent non-substrate inhibitors e.g., dihydrokainate (DHK; 2S,4R)-4MG and 4-Me-L-*trans*-2,4-PDC, were selected to generate a test set (Figure 3.7). Similar to the training set, the criteria for the selection for the test ligands were high potency, relative selectivity, no substrate activity and structural diversity. Conformational space searching for each test ligand was done using randomsearch, and AESOP (or dynamics (Tripos) stochastic methods. Three more databases containing unique test set conformers with the required angles and measures were created. All the conformations of the test ligands were compared against the generated pharmacophore, model A (Figure 3.2) and scored for relative structural similarity using Equation 1. The low scoring function values showed tight alignment of the test ligands against the pharmacophore thus confirming the robustness of the model (Figures 3.13-3.15).

Our non-transportable inhibitor-based model was then used to define putative regions that may distinguish substrates from non-substrates. Three common substrates, L-glutamate, D- and L- aspartate (Table 3) were used in our modeling protocol to generate multifit models as shown in Figures 3.16. As can be seen, these substrates fit extremely well with

the pharmacophore model at low scoring function values. They appear to be virtually indiscernible within the pharmacophore, while the non-substrate bulk seems to protrude toward the periphery. This arrangement of excess bulk caused by the substituents, as well as cyclic carbons, may hinder effective transport as a result of unfavorable energy requirement to assume substrate-like conformation. Moreover, the direct interaction of the substituents with the binding site can influence their transport activity.

Recently, Esslinger et al have synthesized an EAAT3 selective non-transportable inhibitor L-β-benzyl aspartate (L-β-BA). L-β-BA was characterized by the other members of our lab and by Michael Kavanaugh's lab. L-β-BA shows approximately 10-fold greater activity at EAAT3 than EAAT1 or EAAT2 in C17.2 cells and the electrophysiological recordings revealed that it acts as a competitive inhibitor (Esslinger et al., 2005). These findings have been summarized in Figure Table 3.4. Additionally, it should be noted that the *erythro* stereoisomers of both L-β-BOA and L-β-BA have been found to be significantly less potent than their *threo* counterparts (Esslinger et al., 2005; Shimamoto et al., 2000) (Table 3.4). Secondly, whereas L-β-TBOA is relatively more selective for EAAT2 (Shimamoto et al., 1998; Shimamoto et al., 2000), L-β-TBA is more selective for EAAT3 (Table 3.5). This suggests that certain stereospecific interactions may account for differences in the activities of the stereoisomers of L-β-*threo*-BA at EAAT2 and EAAT3. We exploited our EAAT2 specific model in an attempt to identify these differences. To see if any variations could be predicted between EAAT2 and EAAT3 binding sites, we incorporated L-β-*threo*-BA into our model as well as the *erythro*- stereoisomers of both L-β-BA and L-β-BOA. All three compounds were

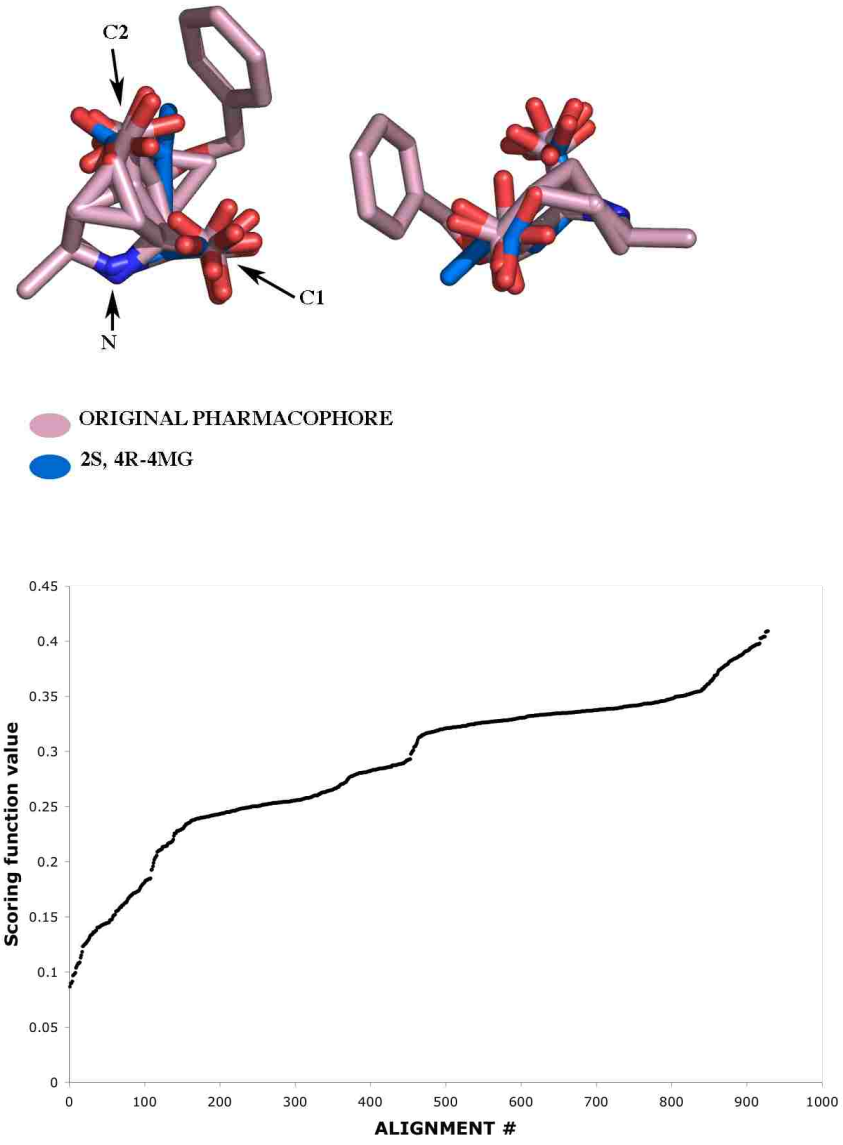
Table 3.3. Inhibitory activity of test set ligands at EAAT1, EAAT2 and EAAT3.



TEST SET						
	cis-4-CH_3-L-*trans*-2,4-PDC		**Dihydrokainate**		**2S,4R-4-MG**	
	K_i (μM)	I_{max} (%)	K_i (μM)	I_{max} (%)	K_i (μM)	I_{max} (%)
EAAT1	14.1	21	-	-	32	71.8
EAAT2	1.8	0	9.2	0	3.1	0
EAAT3	16.6	0	0	0	34	66
# Unique Conformers	88		528		928	
Energy Range (kpm)	8.309-13.401		10.202-25.588		1.741-13.155	

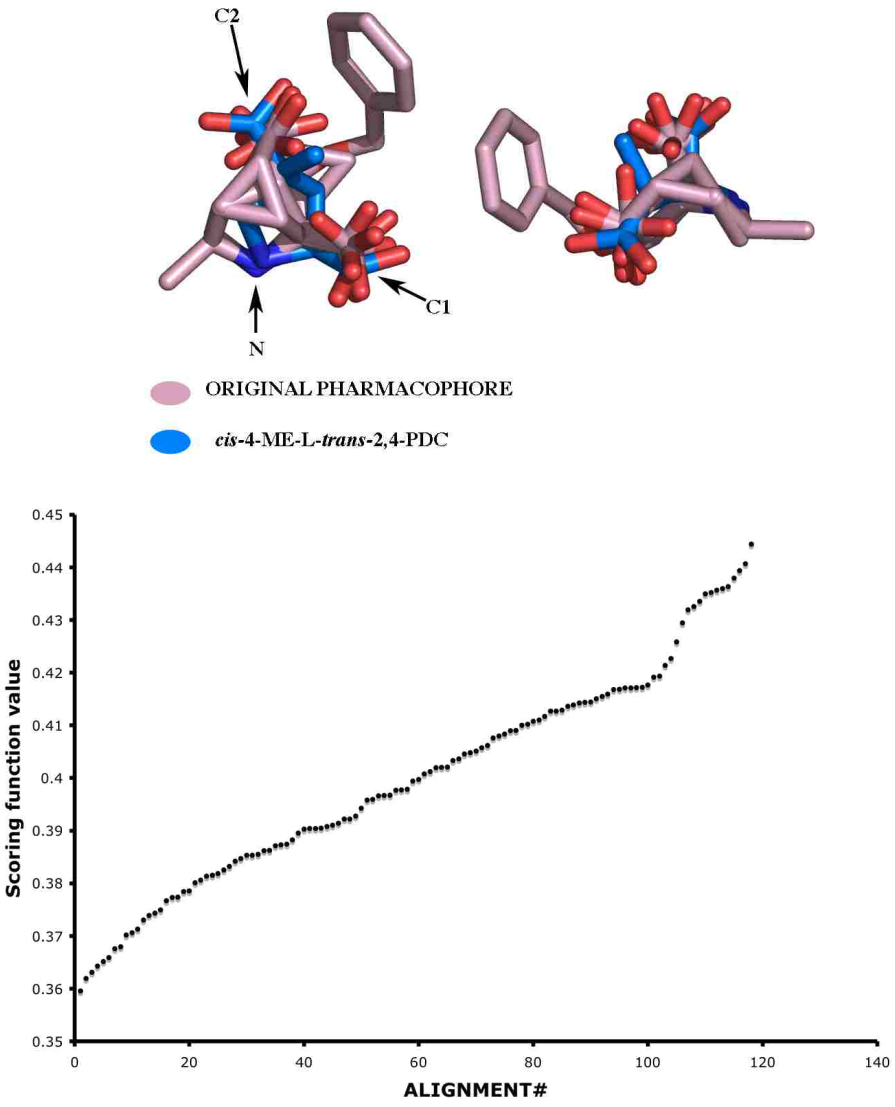
Three EAAT2-preferring non-substrate inhibitors were selected in the test set. The indicated points-of-interest (N, C1, C2) are shared by all the ligands in both the training set and the test set. The number of unique conformers and the energy range are also shown in the table.

Figure 3.13. Comparison of the test set ligand, 2S,4R-4MG, with the pharmacophore model A.



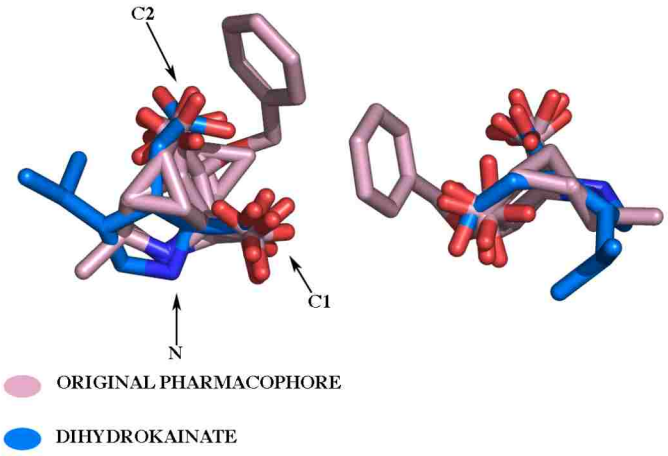
2S,4R-4MG superpositioned on the pharmacophore model A was selected based on the low scoring function value. The points-of-interest are labeled. Two views of the same alignment are shown. The scoring function values are shown for different alignments in the chart.

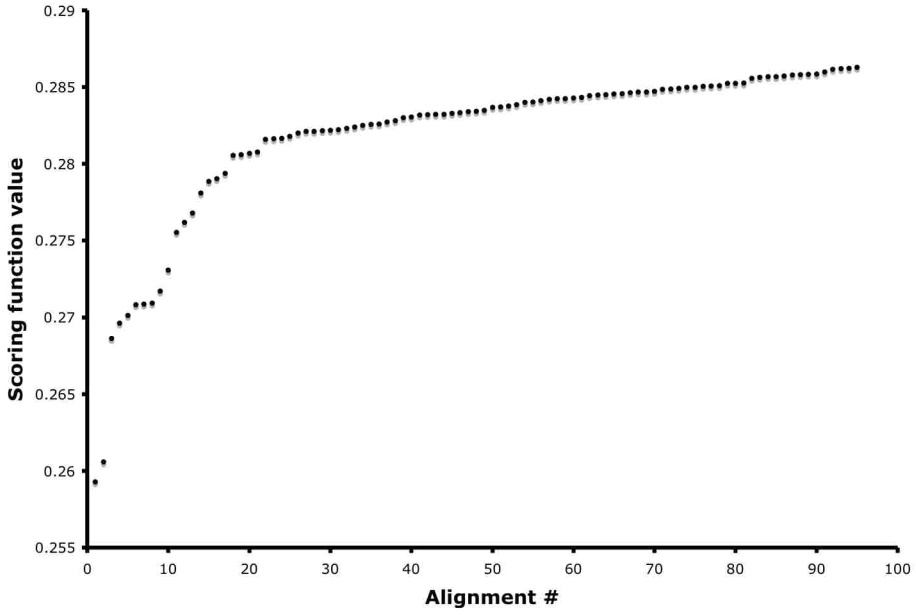
Figure 3.14. Comparison of the test set ligand, *cis*-4-methyl-L-*trans*-2,4-PDC, with the pharmacophore model A.



DHK superpositioned on the pharmacophore model A. The alignment group was selected based on the low scoring function value. The points-of-interest are labeled. Two views of the same alignment are shown. The scoring function values are shown for different alignments in the chart.

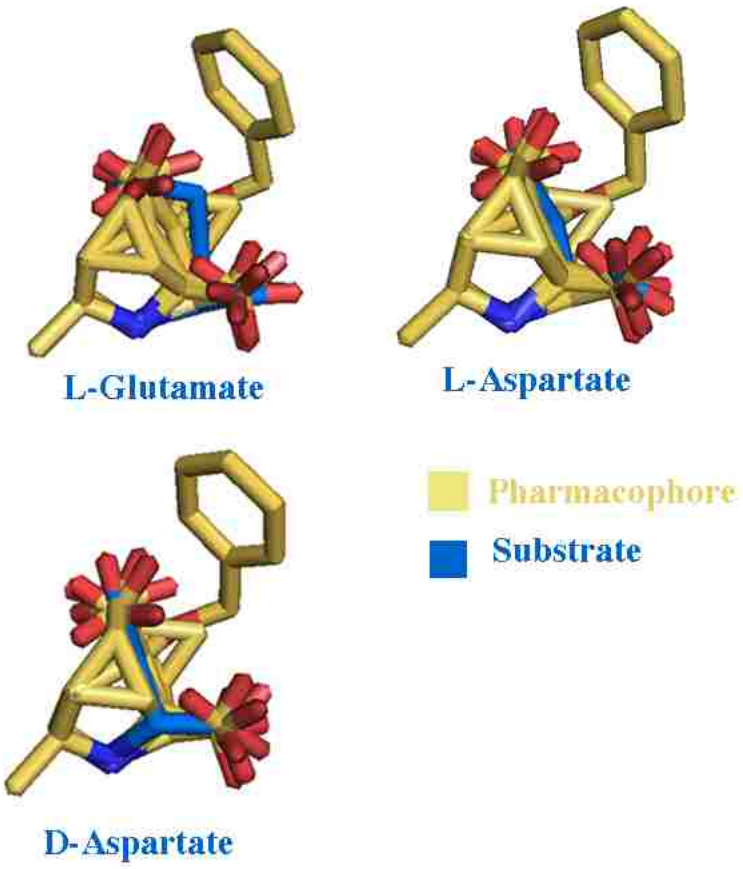
Figure 3.15. Superpositioning of dihydrokainate (DHK) on the EAAT2 binding-site pharmacophore model A.





Based on the low scoring function value, the above alignment was selected. The points-of-interest are labeled. Notice the positioning of the isopropyl group of DHK and the methyl group of *cis*-5-methyl-L-*trans*-2,3-PDC are oriented in the same region.

Figure 3.16. The superposition of L-glutamate, L-aspartate and D-aspartate with the EAAT2 binding site pharmacophore model.



These substrates show almost identical alignment when superpositioned (1 cal spring constant) with the model. The alignment groups are selected based on their low scoring function values.

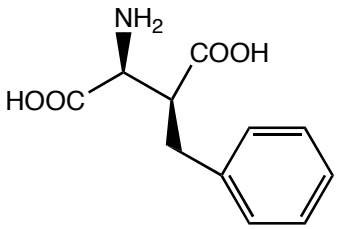
subjected to same rigorous space searching using stochastic methods mentioned above. The resultant molecular databases containing all the unique conformers were compared to the pharmacophore to generate the similarity score using equation 1 (Figure 3.17). The resultant multifit alignments shown in Figure 3.13 were selected on the basis of low scoring function value and visual inspection with CrystalEyes viewer (Figure 3.18). The similar positioning of the lipophilic phenyl ring of both L-β-TBOA and L-β-TBA suggests lipophilic interactions within the binding sites of the transporter and that the *erythro*- stereoisomers may render the phenyl rings inaccessible to this region. This lipophilic region may be common to both EAAT2 and EAAT3. However, subtle differences in the size, location and/or orientation of the aromatic ring with respect to the lipophilic residues in the transporters may exist. It seems likely that the ether 'O' of L-β-TBOA may be participating in the electrostatic interaction within the EAAT2 binding site whereas this interaction may be substituted by a more lipophilic interaction within the EAAT3 binding site thus contributing to the differential effects produced by L-β-TBOA and L-β-TBA.

Chapter 3: Discussion

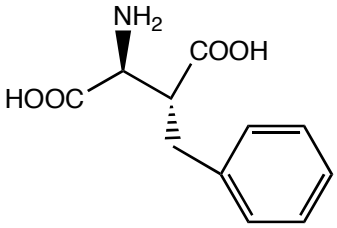
Taking advantage of the availability of selective ligands for EAAT2, we have built a non-substrate inhibitor based EAAT2-specific binding site pharmacophore model (Figure 3.8). The model was derived by calculating 3D structural similarities of multiple training set ligands (Martin, 1998; Perkins and Dean, 1993; Dean and Perkins, 1998). The inclusion of ligands in the training and test sets was based upon structural diversity,

Table 3.4. Inhibitory activity of β-substituted aspartate analogues is EAAT1, EAAT2 and EAAT3.

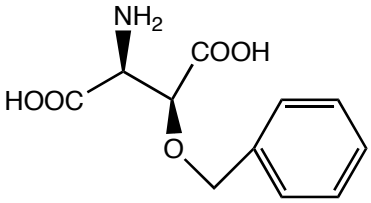
COMPOUND	CONC	EAAT1 ^{3}H-D-Asp Uptake (% of Control)	EAAT2 ^{3}H-D-Asp Uptake (% of Control)	EAAT3 ^{3}H-D-Asp Uptake (% of Control)
L-Aspartate	**100μM**	4	16	15
D,L-β-*threo*-Benzyloxy-Asp	**100μM**	5	2	9
L-β-*threo*-Benzyl-Asp	**100μM**	8	9	1
L-β-*erythro*-Benzyl-Asp	**100μM**	59	48	14



L-β-*threo*-benzyl aspartate (L-β-TBA)



L-β-*erythro*-BA (L-β-EBA)



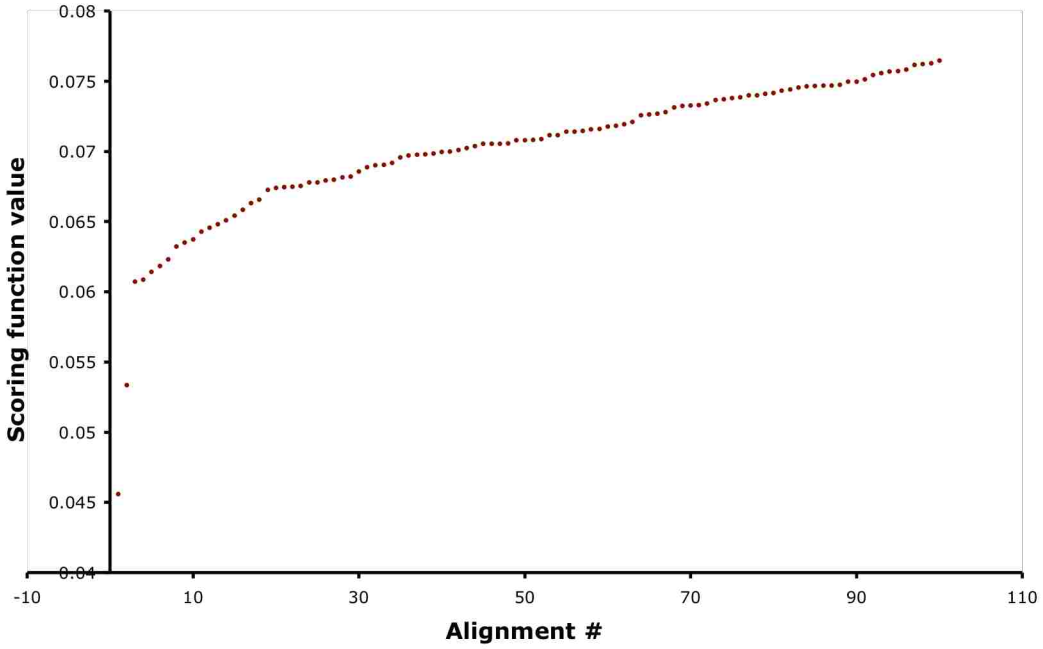
L-β-TBOA

Table 3.5. The inhibitory activity of L-β-TBA and L-β-TBOA at EAAT1, EAAT2 and EAAT3.

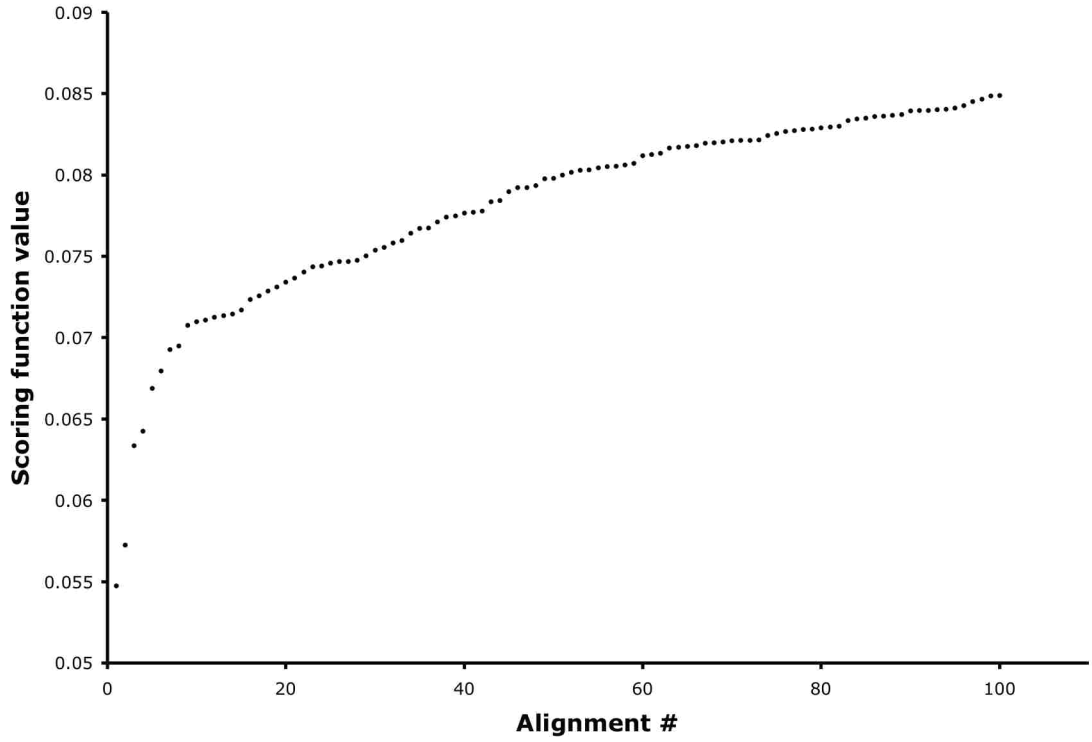
	L-β-TBA	**D,L- β-TBOA**
	K_i (μM)	**K_i (μM)**
EAAT1	8.7	9
EAAT2	10.0	0.2
EAAT3	0.8	-
# Conformers	817	208
Energy Range (KPM)	1.0916-437	1.635-1116.14

Figure 3.17. Charts showing the distribution of alignments groups for the L-β-TBA, L-β-EBA and L-β-EBOA.

A. L-β-TBA



B. L-β-EBA



C. L-β-EBOA

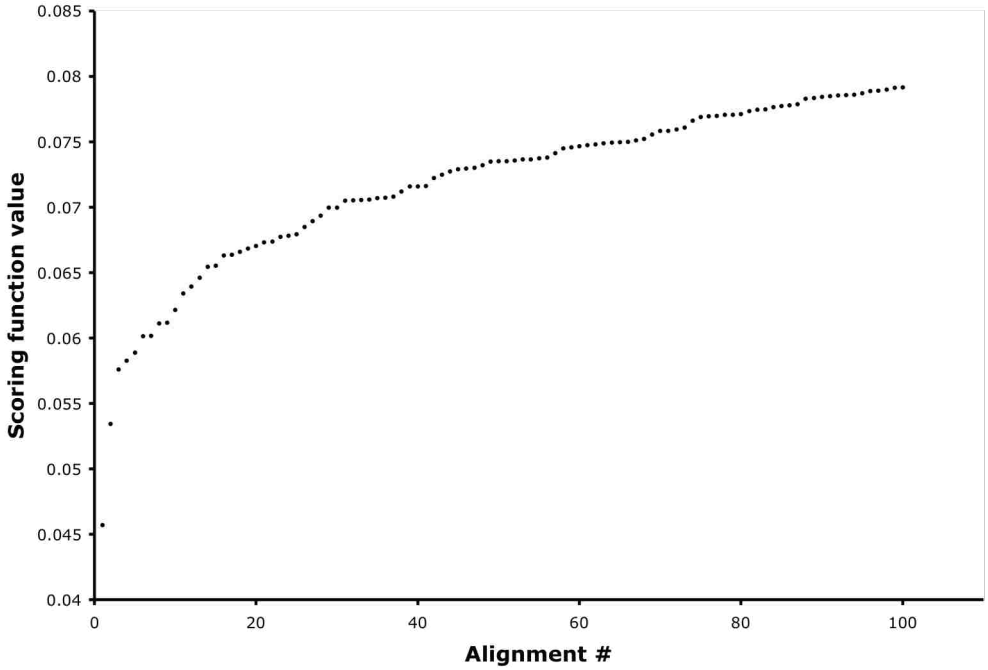
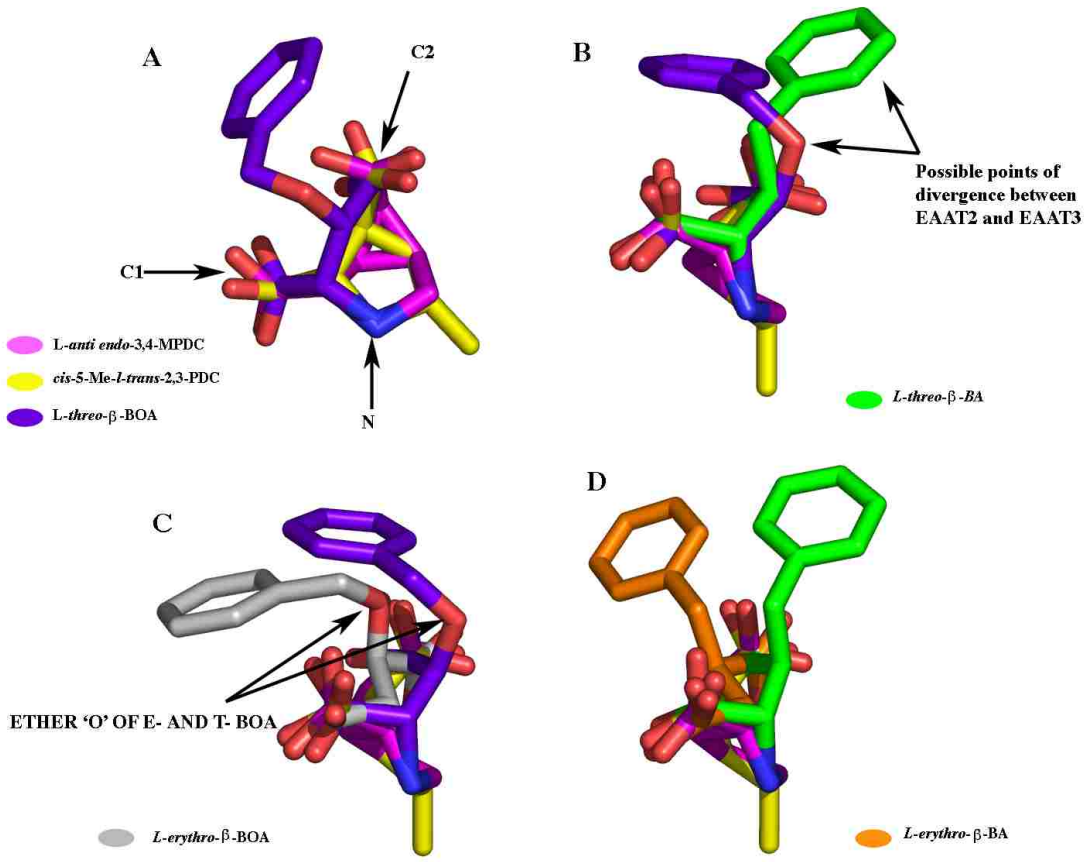


Figure 3.18. Assessment of conformations and activities of L-β-TBOA, L-β-EBOA, L-β-TBA and L-β-EBA against the EAAT2 binding-site pharmacophore.



The pharmacophore model is shown in (A). The superposition (1 cal spring constant) of L-β-TBOA and L-β-TBA with the model (B) suggesting that possible points of divergence between the EAAT2 and EAAT3 pharmacophores may include subtle differences in the size, location and/or orientation of the aromatic ring or in the oxygen atom present in the linking group of L-β-TBOA, but not L-β-TBA. In (C) the

superposition of L-β-*threo*- and L-β-*erythro*-BOA with the model illustrating the better fit of aspartyl backbone of the *threo* diastereomer with other aligned molecules, in addition to the distinct placements of the ether 'O' and the benzyl group that might influence the potency and selectivity for this inhibitor at EAAT2. A similar conclusion is reached regarding L-β-TBA, when the two diastereomers of the L-β-BA are compared.

potency and relative selectivity. Comparison of one conformer of one ligand to all the conformers of each of the three remaining ligands in the training set was based on six selected measures between the common functional groups, e.g., one amino and two carboxylate groups, shared by each ligand. The lower scoring function values for comparison groups represented the least amount of differences amongst the six variable conformer measures in 3D space. Based upon low scoring function values and visual appraisal in stereoview using CrystalEyes viewers, the final model was selected. The intent of this model is to predict structural requirements for the ligand to act as substrate or a non-substrate inhibitor, as well as identify putative points of difference between the EAAT subtypes.

The planar arrangement of the two carboxyl groups and the amino group in 3D space suggests that these regions may identify a critical ligand triad that defines the initial recognition within the binding site. As was demonstrated, both the substrates and non-transportable inhibitor aligned closely on these selected points-of-interest, suggesting that these functional groups may exhibit electrostatic interactions with the complementary residues found within the EAAT binding site and possibly with select ions. Subsequently, other critical ligand structural properties may influence important inhibitor behavior such as: substrate versus non-substrate ligand activity, potency, and EAAT subtype selectivity. Our model defines three additional regions distinct from the carboxyl and amino functionalities within the 3D model space.

One of these regions is occupied by the extended arm of the benzyl group of L-β-TBOA. The presence of amino acid residues near the initial recognition binding site moieties may offer lipophilic interaction with the benzyl group in this region. This idea is further supported by the newly identified ligand, WAY213613 (Dunlop et al., 2005). In the modeling studies by this group, WAY213613, was shown to project its aromatic side chain, which occupies a much larger volume, in the same direction as the benzyl group of L-β-TBOA. Additionally, the orientation of the benzyl group within the binding site may play a crucial role in determining this lipophilic interaction. When the less potent *erythro* stereoisomer L-β-EBOA (Shimamoto et al., 2000) was aligned with our pharmacophore model, the two benzyl groups occupied distinct positions within the binding site with respect to the amino acid and carboxylate triad discussed above (Figure 3.18). The positioning of the benzyl group of L-β-TBOA suggests that this model presents an orientation that is more favorable for complimentary interaction with the residues in the binding domain. It is plausible that the L-β-EBOA benzyl group lipophilic interaction is disrupted or it experiences steric hindrance or both within the binding site.

The test set ligand, DHK, has been shown to be an EAAT2-selective, non-tranportable inhibitor (Arriza et al., 1994). When superpositioned on our pharmacophore model as a validation step, DHK projected its isopropyl group in a distinct region. This isopropyl group has been shown to be an important determinant for activity at glutamate transporter, since 2-carboxy-3-pyrrolidineacetate, in which the isopropyl group is absent, is a weaker inhibitor of glutamate transport , even though it has enhanced binding capacity to KA, AMPA and NMDA receptors (Sonnenberg et al., 1996). The closely

related analogue, L-*trans*-2,3-PDC, has also been shown to be a preferential EAAT2 non-substrate inhibitor (Bridges et al., 1999). The addition of a methyl susbstituent at 5-position of L-*trans*-2,3-PDC also has important consequences on its activity. Whereas, the *cis*-5-methyl addition retains the compound's inhibitory capacity without compromising potency, the *trans*- addition abolishes its activity. Interestingly, in the superposition model, DHK projected its isopropyl side chain in the general vicinity of the methyl group of *cis*-5-methyl-L-*trans*-2,3-PDC. This unique placement of methyl and isopropyl groups suggests a stereospecific lipophilic interaction in this region. It remains less clear whether this area is a distinct lipophilic pocket associated with the EAAT protein.

Another region, that may be important, is the distinct positioning of the cyclopropyl ring of L-*anti*-*endo*-3,4-MPDC in our model . This area may confer a Π-bond like character to this region and that modest structural ligand changes may be tolerated within the complementary binding area. Traditional substrates for EAATs have been mostly acyclic and devoid of steric bulk. It appears that the modification of parent substrate either by addition of substituents or the introduction of cyclic constraint, may hinder effective transport as a consequence of excess bulk and / or substituent-induced ligand conformational changes as demonstrated by 2,3-PDC and methyl glutamate analogues. However, constraining a molecule through the incorporation of cyclic structures can lead to both the substrate and the non-substrate inhibitors. For example, the highly restricted bicyclic analogues of PDC, L-*anti*-*endo*-3,4-MPDC and 2,4-MPDC, have been shown to inhibit glutamate transport in rat forebrain synaptosomes (Esslinger et al., 1998).

However, electrophysiological studies in oocytes revealed that whereas L-*anti-endo*-3,4-MPDC is a non-substrate inhibitor, 2,4-MPDC exhibits excellent substrate acitivity at EAAT2 expressed in oocytes (Esslinger et al., 1998). Therefore, the obvious assumptions regarding the ability of ligands to transport based solely upon the degree of flexibility can be misleading.

The inclusion of substrates into our model was done to identify the plausible properties that separate substrates from non-substrates. The general close fit of L-glutamate, L- and D- aspartates within the pharmacophore at low scoring function values confirms its predictive quality. The protrusion of substituents (e.g., (2S,4R)-4MG, *cis*-4-methyl-2,4-PDC, L-β-TBOA) (Figure 3.16) as well as the cyclic rings (e.g., L-*trans*-2,3 PDC, DHK) (Figures 3.15), towards the periphery may invoke additional interactions with the binding site residues that may preclude the ability of the ligands to be effectively translocated. Thus, enhanced hydrophobic interactions within the binding site, by addition of the lipophilic substituents to the ligand, can increase potency while reducing the substrate activity of the inhibitor.

The novel EAAT3-preferring inhibitor L-β-BA exhibits similar stereochemical trends as L-β-BOA. The *threo*- stereoisomer of L-β-BA is significantly more potent than the *erythro* form. When incorporated within our EAAT2-specific model, the benzyl group of L-β-TBA aligned well with that of L-β-TBOA (Figure 3.18 D). As expected from the analysis of L-β-BOA, the benzyl group of *threo*- and the *erythro*- stereoisomers of L-β-BA occupy distinct regions in our model. Thus, both the EAAT2 and EAAT3 binding

sites may be thought to accommodate the benzyl groups to various extents and offer the lipophilic interaction with the amino acid residues conferring high potencies for respective *threo* stereoisomers. What separates the EAAT subtype-specific potencies of L-β-TBOA and L-β-TBA may be the linking of phenyl group to the aspartate backbone and the interaction with the binding site residues within this region. Additionally, differences in the orientation, size and location of the lipophilic groups can play significant role in determining the selectivity of the inhibitor. The oxygen atom of L-β-TBOA may be involved in electrostatic interactions like hydrogen bonding within the EAAT2 binding site residues. It may be that the corresponding residues within the EAAT3 binding site prefer hydrophobic rather than electrostatic interactions. Hence, selectivity for one EAAT subtype relative to another may be driven by either favorable or unfavorable ligand-protein residue side chain interactions involving the ligand phenyl group and the linking region.

Recently, John Gerdes' research group has also developed an EAAT3 homology model using the coordinates from the crystal structure of Gltph sequence (Yernool et al., 2004). The studies were performed at the Molecular Computational Core Facility at the University of Montana. The investigation utilized a Linux (Redhat Enterprise 3) workstation (dual 3.0 GHz processors, 2 GB memory) employing SYBYL 7.0 (Tripos, Inc.; St. Louis, MO) and related Bioploymer and FlexX software suites. Submission of the alignment to SwissModel provided the homology model. The docking studies were subsequently done with the hEAAT3 homology model to define substrate space coordinates. Utilizing this model, we superpositioned our EAAT2 pharmacophore on the

defined substrate space coordinates in an attempt to compare the binding site interactions. The positioning of the benzyl group of L-β-TBOA, as configured in our pharmacophore, suggested a possible steric clash with the EAAT3 transmembrane domain 7 (Figure 3.19). However, rotation of L-β-TBOA in our model by 180^0, while maintaining the 3-point consistency of the functional groups, orients its benzyl group in the general direction of the HP2 loop. This later orientation of L-β-TBOA is more appropriate considering the lipophilic nature of the HP2 loop. Moreover, it has been reported that the HP2 loop can undergo a conformational change upon binding of ligands and thus, acts as an extracellular gate (Grunewald et al., 1998; Grunewald and Kanner, 2000; Slotboom et al., 1999; Slotboom et al., 2001; Zarbiv et al., 1998; Seal and Amara, 1998; Seal et al., 2000).

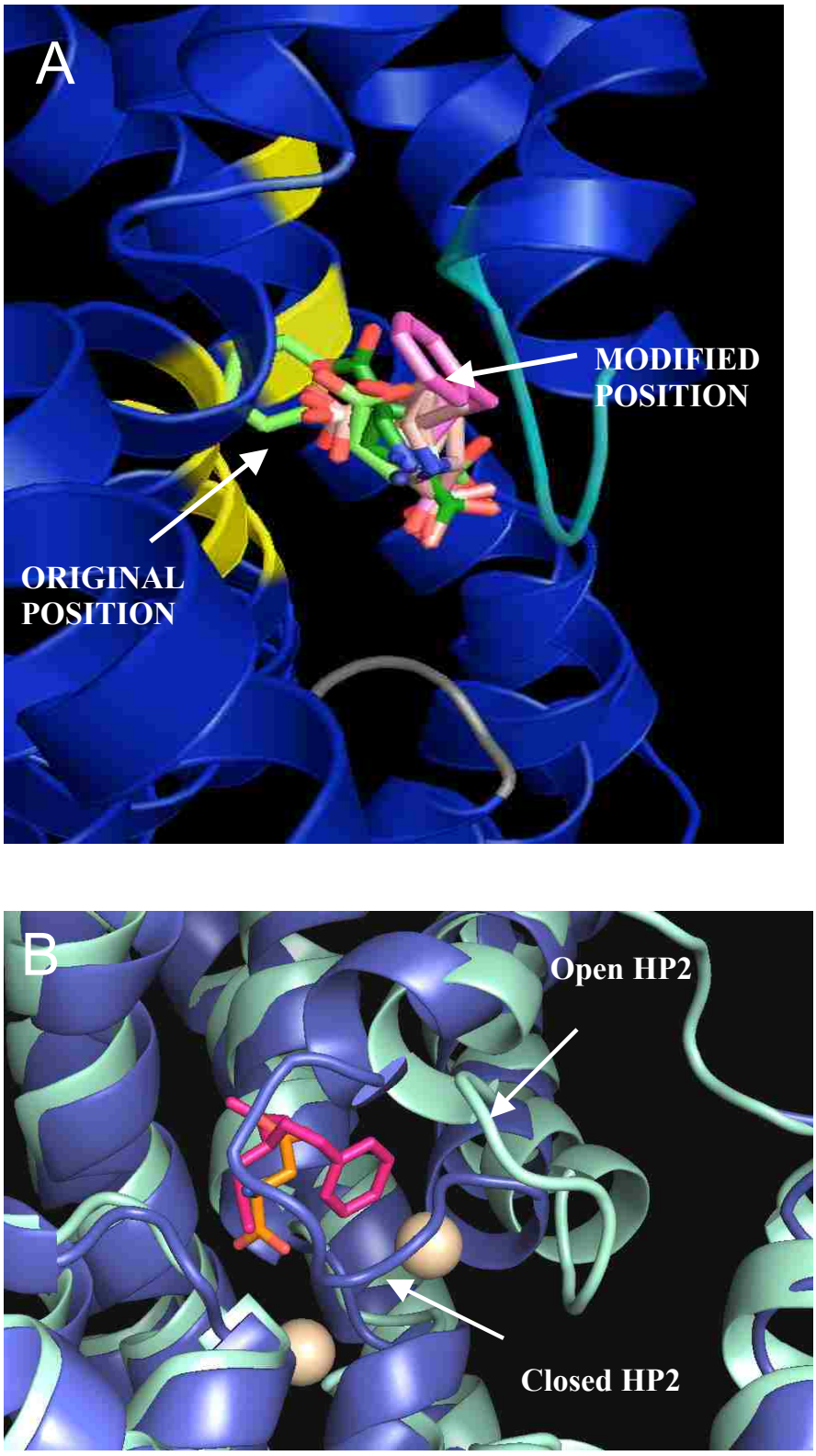
In the last few months, another set of high resolution crystal structures of EAAT bacterial homologue GLT_{PH} were published with either the substrate, L-aspartate, or the non-transportable inhibitor, L-β-TBOA, bound to the transporter (Boudker et al., 2007). This provided an opportunity to compare our pharmacophore with the L-β-TBOA from the crystal structure. The amino nitrogen, the distal and proximal carboxylate carbons, the benzyloxy oxygen, as well as the carbon backbone matched well with each other. The six measures (3 angle and 3 distances), as predicted from our model and the L-β-TBOA bound in the crystal structure, were found to be almost identical.

In both instances, the benzyl groups of L-β-TBOA appears to project toward the HP2 loop. The two configurations of L-β-TBOA did, however, differed from each other only with respect to the rotation of benzyl group about the ether oxygen atom (Figure 3.19).

The differences are likely attributable to the fact that in our model, L-β-TBOA was docked on the "closed" or the substrate-bound form rather than the "open" non-substrate bound form. The results suggest that the phenyl group of L-β-TBOA seems to interact with helical hairpin 2 (HP2) loop. The possibility of HP2 loop acting as an extracellular gate is compelling. Boudker et al propose that the binding of two sodium ions and L-aspartate causes HP2 loop to close, thereby permitting the conformational change needed for the subsequent transport (Boudker et al., 2007). The binding of L-β-TBOA prevents HP2 from closing, thus locking the transporter in an 'open' state.

Both, our pharmacophore model and the crystallographic data, suggest that the functional groups and the carbon backbone of the substrates occupy very similar, if not identical, regions. Thus, it is possible that subtype-selectivity and substrate activity resides in the subtle differences in the size and orientation of the lipophilic substitutions that can be allocated to the template. Exploiting these subtle differences among the binding sites of EAAT subtypes using these compounds' templates can lead to development of ligands with highly selective binding profiles. With the development and refinement of specific models for each subtype, design of more selective substrates and non-transportable inhibitors is possible. Identifying and incorporating important functional domains of novel ligands into evolving models will significantly improve our understanding of the physiology and pathophysiology attributed to these transporters.

Figure 3.19. Positioning of L-β-TBA docked on the binding site of EAAT3 homology model.



A. The alignment of L-β-TBA (superpositioned with the pharmacophore model) with the defined substrate space coordinates within the EAAT3 homology model. The original positioning of the phenyl ring of L-β-TBA (green) suggests a possible steric clash with the EAAT3 transmembrane domain 7. However, rotation of L-β-TBA in our model by 180^0 (pink), while maintaining the 3-point consistency of the functional groups, orients its benzyl group in the general direction of the HP2 loop. **B**. The positioning of L-β-TBOA as reported in the crystal structure by Boudker et al. 2007.

Chapter 4: Characterization of EAAT Modulation by Neuroactive Steroids

Introduction:

Modulation of EAATs: The glutamate transporter system is highly regulated and one that is modulated at different levels. For example at the genomic level, certain compounds, including: β-lactam antibiotics, injury-induced growth factors, retinol and corticosterone have been shown to alter transcription and translation of the transporters (Rothstein et al., 2005; Tian et al., 2007; Su et al., 2003; Figiel et al., 2003; Schlüter et al., 2002; O'Shea et al., 2006; Thorlin et al., 1998). EAATs can be also be regulated by changes in trafficking (Gonzalez and Robinson, 2004; Hughes et al., 2004; Duan et al., 1999). In addition, certain molecules including EAAT interacting proteins (Jackson et al., 2001; Lin et al., 2001), zinc (Mitrovic et al., 2001) and arachidonic acid (Zerangue et al., 1995), appear to influence their function by allosteric modulation (Vandenberg et al., 2004). A review on the modulation of these transporters by genomic and the trafficking mechanisms is presented in Chapter 1.

Allosteric Modulation of glutamate transporters:

Zinc: Zinc has been suggested to exhibit differential effects at the EAAT subtypes (Mitrovic et al., 2001; Vandenberg et al., 1998a). While Zn^{2+} was shown to modulate the activities of EAAT1 and EAAT4 expressed in *Xenopus laevis* oocytes, it appears to have no effects on EAAT2 or EAAT3. The binding of Zn^{2+} ion to EAAT1 inhibited the transport of glutamate in a non-competitive fashion. In the case of EAAT4, however,

arachidonic acid was shown to selectively inhibit the chloride conductance, with little effect on the transport of glutamate. The effects of zinc were found to be fully reversible and the Zn^{2+}-binding sites have been identified in the glutamate transporters using site-directed mutagenesis (Mitrovic et al., 2001; Vandenberg et al., 1998a).

Polyunsaturated fatty acids (PUFAs): Another small molecule, arachidonic acid, has been shown to differentially modulate the activities of EAAT1, EAAT2 (Zerangue et al., 1995) and EAAT4 (Fairman et al., 1998; Tzingounis et al., 1998; Poulsen and Vandenberg, 2001). While arachidonic acid, at micromolar concentrations, inhibited the EAAT1-mediated glutamate uptake by reducing the maximal transport rate by about 30%, it increased the apparent affinity of glutamate for EAAT2 more than 2-fold when expressed in oocytes and HEK293 cells (Zerangue et al., 1995). In a similar vein, arachidonic acid was reported to reduce V_{max} for glutamate uptake in salamander Müller cells, which expresses EAAT1 as the major transporter, by affecting membrane characteristics (Barbour et al., 1989). However, arachidonic acid was also reported to non-competitively inhibit EAAT2 (GLT1) in reconstituted system in which the purified transporter was incorporated into liposomes (Trotti et al., 1995). Taken together, these results suggest that the effects of arachidonic acid may be dependent upon the cell-type in which the transporter is expressed, and/or the make-up of lipids surrounding it.

Studies on EAAT4 concluded that while there is no change in the uptake of glutamate in the presence of arachidonic acid, it was found to activate an uncoupled proton current associated with glutamate-bound EAAT4 (Fairman et al., 1998; Tzingounis et al., 1998;

Poulsen and Vandenberg, 2001). This effect was attributed to the binding of arachidonic acid directly to EAAT4. Additionally, the cyclo-oxygenase inhibitor, niflumic acid (Poulsen and Vandenberg, 2001) and other polyunsaturated fatty acids (PUFAs), e.g., docosahexaenoic acid (DHA) and linolenic acid (Fairman et al., 1998) resulted in similar activation of this proton current. In addition to the effects produced by simultaneous application, these compounds have the ability to differentially alter glutamate transport following longer times of exposure (Berry et al., 2005). For example, preincubation with DHA for 10-40 minutes was reported to modulate the activities GLT-1, GLAST and EAAC1 via different mechanisms in HEK cells. In the instance of GLT-1 and EAAC1, DHA (100-200μM) appears to stimulate D-[^{3}H]aspartate uptake ~72% and 45% respectively via a mechanism requiring extracellular Ca^{2+} and involving CaM Kinase II and PKC, but not PKA. In contrast, the inhibitory effect (~40%) on GLAST does not require extracellular Ca^{2+} and does not involve CaM kinase II, PKC or PKA (Berry et al., 2005).

The differential regulation of EAATs supports the concept that the subtypes may play important individual roles in controlling extracellular glutamate concentrations needed for signaling. In the present work, we have identified and characterized a potentially novel site at which the EAATs may be differentially regulated. The compounds that were used to characterize this site were neuroactive steroids, including pregnenolone sulfate (PREGS).

Neuroactive Steroids: The nervous system is a target for two different pools of steroids, one coming from the peripheral glands (i.e., steroid hormones) and the second one originating directly in the nervous system (i.e., neurosteroids) (Agis-Balboa et al., 2006; Koenig et al., 1995; Plassart-Schiess and Baulieu, 2001). Central and peripheral nervous systems have inherent enzymatic capacity to synthesize various neurosteroids from cholesterol or other steroidal precursors (Baulieu, 1998; Corpechot et al., 1981; Corpechot et al., 1983; Corpechot et al., 1993; Liere et al., 2000). Furthermore, certain steroids remain in the nervous system long after adrenalectomy or gonadectomy (orchidectomy) (Corpechot et al., 1981; Corpechot et al., 1983; Corpechot et al., 1993; Liere et al., 2000). "Neuroactive steroids" is the general term that encompasses all the steroids present in the brain. They may be derived by *in situ* synthesis, obtained from the peripheral hormones, or converted by enzymatic activation in metabolites which are more active and in some cases utilize a different mechanism of action (Melcangi and Panzica, 2006; Paul and Purdy, 1992) [Paul et al 1992; Melcangi et al 2006].

Neurosteroids are synthesized from cholesterol by a series of enzymatic reactions mediated both by P450 and non-P450 enzymes (Mellon and Griffin, 2002; Mellon et al., 2001; Robel and Baulieu, 1995). The biosynthesis of steroids and neurosteroids requires the movement of cholesterol from the outer to the inner mitochondrial membranes where cholesterol side-chain cleavage enzyme, cytochrome $P450_{scc}$ (CYP11A1), resides and converts cholesterol into pregnenolone, the precursor to other neurosteroids (Mellon and Griffin, 2002; Mellon et al., 2001). This dynamic process is modulated by both the control of the intrinsic enzymatic activity of P450scc and by substrate availability. For

this reason, cholesterol transport within the mitochondrion has emerged as the key regulation point for steroidogenesis. Peripheral-type benzodiazepine receptor (PBR), along with steroidogenic acute regulatory protein (StAR), facilitates the efficient production of steroid hormones by regulating the translocation of cholesterol across the mitochondrial membranes (Jefcoate, 2002; Papadopoulos, 2004; Papadopoulos et al., 1997). The brain contains additional steroid metabolizing enzymes, including sulfonyltransferases and sulfohydroxylases, which convert classic hormones to a variety of sulfated neuroactive compounds. To maintain and regulate the effects of neuroactive steroids, the steroidogenic enzymes in the CNS and PNS are regulated during development. Moreover, their regulation is region and cell-specific.

Neuroactive steroids exert their effects on the brain either through activation of intracellular steroid receptors (genomic pathway) or via non-genomic route (McEwen, 1994; Plassart-Schiess and Baulieu, 2001). The genomic effects are characterized by a delayed onset and prolonged in duration, while non-genomic effects are typically rapid in onset and shorter in duration (McEwen, 1994).

Genomic Effects: Steroid hormones that are synthesized in the periphery can cross the blood-brain barrier, and can function at the genomic level to produce changes in mood and behavior. These effects develop relatively slowly (over minutes to hours), and can persist long after the disappearance of the steroid from the brain (McEwen, 1991c; McEwen, 1994).

These effects are mediated by the receptors distributed throughout the brain that are present on both neurons and glia (McEwen, 1991c; McEwen et al., 1986) (McEwen et al., 1986; McEwen, 1991a; McEwen et al., 1983; O'Keefe and Handa, 1990). The steroid hormone receptor superfamily consists of a large number of genes. It includes receptors for the steroids, estrogen (ER), progesterone (PR), glucocorticoid (GR), mineralocorticoid (MR), and androgen (AR) as well as the receptors for thyroid hormone (TR), vitamin D (VDR), retinoic acid (RAR), and 9-*cis* retinoic acid (RXR), and ecdysone (EcR) (Tsai and O'Malley, 1994; Evans, 1988). This superfamily is characterized by a unique modular structure with receptors divided into several domains (Tsai and O'Malley, 1994; Beato, 1989; Evans, 1988; Fuller, 1991). They contain a conserved 66-residue DNA-binding domain and a conserved 240-residue hormone-binding domain. The role of the hormone-binding domain in an intact receptor is to prevent the DNA-binding domain from interacting with DNA unless hormone is bound. The amino-terminal domain, which is conserved, enables a receptor to interact with other transcriptional regulators.

Progestins, estrogens, androgens, and corticosteroids are capable of modifying brain functions and behaviors by mechanisms that involve the classic genomic model for steroid action (McEwen et al., 1983). In this model, steroid hormones must enter target cells to act. It is thought that, because of their lipophilic nature, free steroid hormones enter the target cells primarily by passive diffusion through the cell membrane. However, the evidence for the active transport via membrane transporters (Chen and Farese, 1999; Thompson, 1995) as well as receptor-mediated endocytotic mechanisms is accumulating

(Kralli et al., 1995; Nykjaer et al., 1999; Adams, 2005; Hammes et al., 2005). Hormones such as estradiol, progesterone, testosterone, and cortisol traverse the plasma membrane and bind first to specific receptor proteins in the cytosol. The hormone-receptor complexes (activated receptors) then migrate to the nucleus, where they bind to specific DNA sequences called hormone response elements (HREs) and regulate the expression of nearby genes. The expression of these genes is consequently altered resulting in promotion (or suppression) of transcription.

Non-genomic effects: The rapid effects of steroids, which occur within seconds or a few minutes (Brann et al., 1995; McEwen, 1991c) are not compatible with slower genomic mechanisms involving transcription events (Beato, 1989). The non-genomic effects of neuroactive steroids are produced mainly via an action on membrane proteins. The most thoroughly characterized membrane targets have been $GABA_A$ and NMDA receptors. Pharmacological characterizations have demonstrated that both sulfated, as well as non-sulfated neuroactive steroids, act upon these receptors (Gibbs et al., 2006).

$GABA_A$ receptor modulation by neuroactive steroids: Whereas certain non-sulfated neuroactive steroids seem to potentiate the $GABA_A$ receptor function, their sulfated counterparts inhibit these receptors (Park-Chung et al., 1999). For example, 5α-pregnan-3α-ol-20-one (3α,5α-THPROG or allopregnanolone) and 5β-pregnan-3α-ol-20-one (3α,5β-THPROG or pregnanolone) potently prolong the GABA-mediated inhibitory post-synaptic currents at synapses between rat hippocampal neurons in cultures, oocyte expression system and primary chick spinal cord neurons (Park-Chung et al., 1999;

Harrison et al., 1987). In contrast, PREGS, DHEAS, as well as the 3α and 3β isomers of pregnanolone sulfate, inhibit the GABA-induced currents by allosteric modulation of $GABA_A$ receptors at micromolar concentrations (Majewska et al., 1990; Majewska et al., 1988; Park-Chung et al., 1999). The endogenous progesterone metabolites 3α,5α-THPROG and 3α,5β-THPROG and the deoxycortisone metabolite 5α-pregnan-3α-21-diol (3α,5α-THDOC) are potent stereoselective positive allosteric modulators of $GABA_A$ receptors (i.e., nanomolar concentrations) ("GABA modulatory effect") (Callachan et al., 1987; Peters et al., 1988; Lambert et al., 1995). At relatively higher concentrations (nanomolar to low micromolar) these steroids directly activate the $GABA_A$ receptor-channel complex ("GABA mimetic effect") (Callachan et al., 1987; Shu et al., 2004) at a distinct site from the GABA binding site (Ueno et al., 1997). However, the potency varies widely and is determined by the neuron-type, as well as by subunit composition of $GABA_A$ receptor (Harney et al., 2003; Vicini et al., 2002; Cooper et al., 1999; Brussaard et al., 1997; Koksma et al., 2003; Belelli et al., 2002).

NMDA receptor modulation by neuroactive steroids: In the instance of NMDA receptors, sulfated neuroactive steroids appear to be more active than the non-sulfated ones. The sulfated neurosteroids, PREGS and DHEAS, have been shown to be positive modulators of NMDA receptors at micromolar concentrations (Wu and Chen, 1997; Wu et al., 1991). On the other hand PREGS was shown to inhibit the responses to AMPA and kainate (Wu and Chen, 1997; Wu et al., 1991). The analogs pregnanolone sulfate and epipregnanolone sulfate, which differs from PREGS primarily by the lack of a C-5 – C-6 double bond, inhibit the NMDA response of chick spinal cord neurons. Surprisingly, the

pharmacological studies showed that PREGS and epipregnanolone sulfate do not compete for a common site (Park-Chung et al., 1997). The interactions of steroids with NMDA receptors have been suggested to be allosteric in mechanism (Bowlby, 1993). The non-sulfated neurosteroids, e.g., PREG and pregnanolone are without any modulatory activity at NMDA receptors, which suggests that the negative charge at the C-3 position may be important for ligand-receptor interaction (Weaver et al., 2000).

Sigma$_1$ receptor modulation by neuroactive steroids: In addition, the direct action on the activities on both NMDA and $GABA_A$ receptors, neuroactive steroids have been shown to indirectly modulate these receptors by their action on sigma$_1$ (σ_1) receptors (Monnet and Maurice, 2006).

Pharmacotherapeutic potential of Neurosteroids: Based upon the widespread effects that the neuroactive steroids have on neurotransmission, it is not surprising that their regulation is associated with various physiological and pathophysiological conditions, including stress, pregnancy, neural development and ageing (Paul and Purdy, 1992; Schumacher et al., 2003). In addition to applications in anaesthesia, lower doses of steroids are found to produce anxiolytic, sedative and hypnotic effects (Eser et al., 2006; Gasior et al., 1999; Rupprecht, 2003; Goodchild et al., 2001). Data from preclinical and clinical studies also support the potential efficacy of neuroactive steroids as a novel class of drugs for the therapeutic management of epilepsy, insomnia and drug dependence (Gasior et al., 1999; Gee et al., 1995; Rupprecht, 1997; Rupprecht et al., 1996).

Effects of steroids on glutamate transporters: In addition to their action on receptors, certain steroids including gonadal steroids and corticosteroids have also been suggested to play a regulatory role on the glutamate transporters. These include the upregulation of both EAAT1 and EAAT2 mRNA, protein expression and activity in cultured primary astrocytes following the administration of estrogen for 72hours (Pawlak et al., 2005). This effect was sensitive to ICI 182,780 treatment suggesting estrogen action through nuclear estrogen receptor. The synthetic glucocorticoid, dexamethasone, also produced a marked increase of EAAT2 (GLT-1) transcription and protein levels in cortical astrocytes, whereas EAAT1 (GLAST) expression remained unaffected (Zschocke et al., 2005). Up-regulation of GLT-1 expression was accompanied by an enhanced glutamate uptake, which could be blocked by the specific GLT-1 inhibitor dihydrokainate. The promoting effect of dexamethasone on GLT-1 gene expression and function was abolished by the GR antagonist mifepristone. The stress hormone, corticosterone, has also been reported to regulate GLT-1 expression in the rat hippocampus (Autry et al., 2006). GLT-1 mRNA and protein are upregulated in the hippocampus of ADX, increases that were reversed with administration of physiological levels of GCs, suggesting that basal levels of GCs provide tonic inhibition of GLT-1 mRNA and protein expression. In the high-dose corticosterone paradigm, GLT-1 protein was increased throughout the hippocampus. It has been shown that glucocorticoids like corticosterone and dexamethasone, but not non-glucococorticoids, are capable of producing a rapid (within 15 min), specific and transient (35-45 min) rise (~155-160%) in glutamate levels in hippocampus *in vivo*. These effects are attributed to non-genomic mechanism of action (Venero and Borrell, 1999).

In the present work, we report the specific modulation of the EAATs by certain sulfated neuroactive steroids using C17.2 cells overexpressing EAAT1 and rat primary astrocyte cultures. We find that simultaneous application of neuroactive steroid pregnenolone sulfate (PREGS) alters the ability of EAAT1 to transport atypical substrates like D-aspartate and L-cysteine.

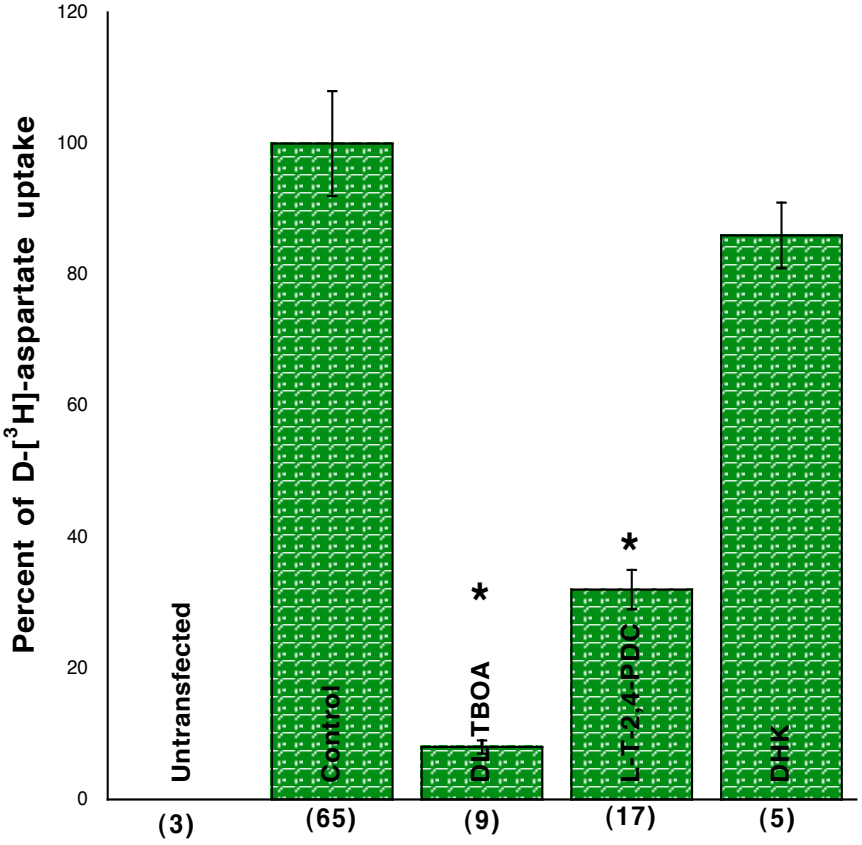
Chapter 4: Results

Pregnenolone sulfate (PREGS) increases the uptake of ^{3}H-D-Asparate in C17.2 cells expressing EAAT1

The uptake of D-[^{3}H]-aspartate by C17.2 cells (Snyder et al., 1992; Snyder et al., 1995) expressing pAM/CAG-hEAAT1 (Esslinger et al., 2005) 24-hour post-transfection is shown in Figure 4.1. D-Aspartate is routinely used to quantify EAAT activity, as it is a non-metabolizable substrate of the transporter. The control rates of uptake of D-aspartate at 10μM were 102±8 pmol/min/mg protein (mean ± s.e.m., n = 65). The C17.2 cells do not show any inherent Na^{+}-dependent D-[^{3}H]-aspartate uptake activity, as was illustrated by the untransfected cells. This uptake was predictably blocked by non-selective EAAT inhibitors like L-β-*threo*-benzyloxy aspartate (L-β-TBOA) (Shimamoto et al., 1998), L-*trans*-2,4-pyrrolidine dicarboxylate (L-*trans*-2,4-PDC) (Arriza et al., 1997) but not by the EAAT2-selective inhibitor dihydrokainate (DHK) (Arriza et al., 1994).

When pregnenolone sulfate (PREGS) at 100μM was similarly tested as a competitive inhibitor, it was found to increase rather than decrease the uptake of D-[^{3}H]-aspartate. As

Figure 4.1. Effect of EAAT inhibitors on the uptake of D-[^{3}H]-aspartate in C17.2 cells transfected with pAM/CAG-hEAAT1.



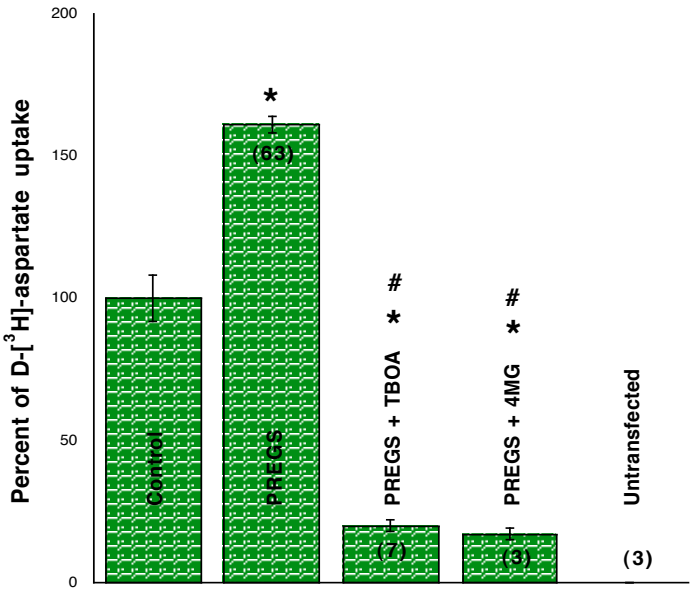
The uptake of D-[^{3}H]-aspartate was measured in C17.2 cells transfected with pAM/CAG-hEAAT1 in the presence and absence of known EAAT inhibitors. The untransfected cells do not show any Na^+-dependent uptake (column 1). Control uptake for 10µM D-[^{3}H]-aspartate was 102 ± 8 pmol/min/mg protein (mean ± S.E.M.). The inhibitor concentrations were 100µM for DL-TBOA and L-t-2,4-PDC, 250µM for DHK. The data are the mean ± S. E. M. of n individual experiment each performed in duplicate. * $p<0.0001$.

reported in Figure 4.2, it increased the Na^+-dependent uptake of ^{3}H-D-aspartate to 161 ± 3% of control (mean ± s.e.m., n = 63). Significantly, the PREGS (as well as all the inhibitors) were added simultaneously with D-aspartate. This uptake is EAAT1-mediated, as it is blocked by non-selective EAAT inhibitors like L-β-TBOA and (2S,4R)-4-methyl glutamate ((2S,4R)-4MG) (Vandenberg et al., 1997). Untransfected cells do not exhibit any uptake activity either in the presence or the absence of PREGS.

Only selective sulfated neuroactive steroids stimulate the uptake of D-[^{3}H]-aspartate by C17.2 cells expressing EAAT1

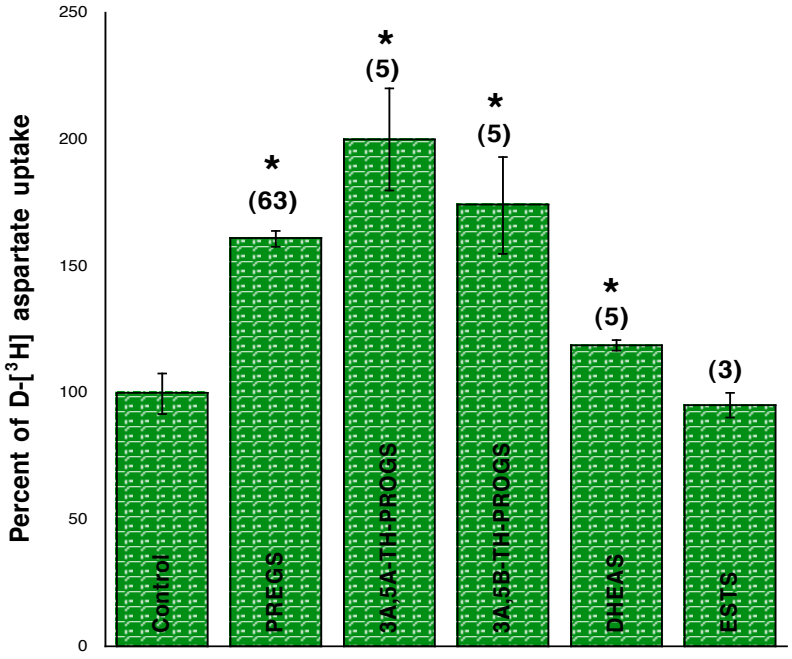
To determine what other steroids may effect the uptake by EAAT1 in C17.2 cells, we screened a number of steroids in different salt forms. These steroids were selected based on their activities at different receptors present within the brain. Thus, DHEAS has been found to have positive modulatory effects on glutamate NMDA receptors and negative modulatory effects on GABA receptors (Wu et al., 1990; Wu et al., 1991; Belelli and Lambert, 2005). The closely related steroids 3α,5α-TH-PROGS (allopregnanolone sulfate) and 3α,5β-TH-PROGS (pregnanolone sulfate), on the other hand, exhibit inhibitory effects on NMDA receptors, as well as $GABA_A$ receptors (Park-Chung et al., 1999; Park-Chung et al., 1994; Park-Chung et al., 1997). When tested on C17.2 cells expressing EAAT1, the structurally related steroids, 3α,5α-TH-PROGS (200±20 % control, n = 5) and 3α,5β-TH-PROGS (174±19 % control, n =5) produced similar effects on D-[^{3}H]-aspartate uptake (Figure 4.3). Interestingly, only minimal changes in activity

Figure 4.2. Potentiation of EAAT1-mediated D-[^{3}H]-aspartate by pregnenolone sulfate (PREGS)



The uptake of D-[^{3}H]-aspartate was measured in C17.2 cells transfected with pAM/CAG-hEAAT1 in the presence and absence of 100µM pregnenolone sulfate (PREGS). The untransfected cells do not show any Na^+-dependent uptake in the presence of PREGS. Control uptake for 10µM D-[^{3}H]-aspartate was 102 ± 8 pmol/min/mg protein (mean ± S.E.M.). The inhibitor concentrations were 100µM for DL-TBOA and 2S,4R-4MG. * and # represent comparison to the control and PREGS treatment respectively. The numbers in brackets represent n. $p < 0.05$.

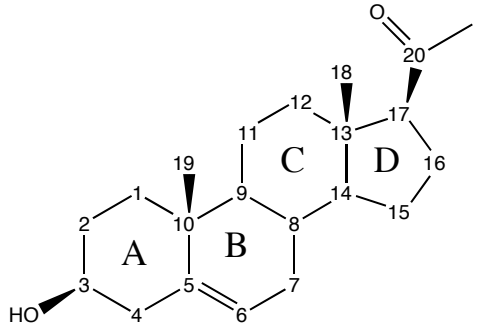
Figure 4.3. Potentiation of EAAT1-mediated D-[^{3}H]-aspartate uptake by sulfated steroids in C17.2 cells.



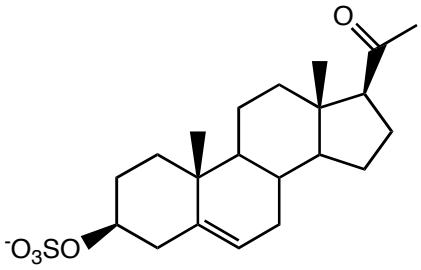
The uptake of D-[^{3}H]-aspartate in C17.2 cells transfected with pAM/CAG-hEAAT1 in the presence and absence of 100µM sulfated steroids. * represents statistically significant values ($p < 0.05$) as compared to the control. The numbers in brackets represent n.

were seen with the addition of dehydroepiendrosterone sulfate (DHEAS), a steroid having similar effects as PREGS on NMDA receptors, GABA receptors and VGLUTs. Other sulfated steroids like 1,3,5(10)-estratrien-3-ol-17-one sulfate (ESTS) failed to effect the uptake of D-[^{3}H]-aspartate by EAAT1. Whereas all the sulfated steroids tested possess a sulfate group at C-3, they differ in the C-17 substitution and the degree of unsaturation (Figure 4.4). PREGS and pregnanolone analogs possess acyl group at C-17, while DHEAS and ESTS have oxygen atom at C-17. The more potent activity observed with PREGS, 3α,5α-TH-PROGS and 3α,5β-TH-PROGS suggests that the acyl side chain at C-17 of these steroids may be important for effective interaction with the target site. The negative charge at C-3 as well as the C-17 substitution have been identified to play critical roles in interaction of steroid with other proteins, including $GABA_A$ receptors (Hosie et al., 2006), estrogen receptors (Brzozowski et al., 1997), and sex hormone-binding globulin (SHBG) protein (Grishkovshaya et al., 2000). The replacement of sulfate (SO_4^{2-}) with another negatively charged group like hemisuccinate at C-3 position has been shown to retain the activity of the corresponding neuroactive steroids at NMDA (Weaver et al., 2000) and $GABA_A$ receptors (Park-Chung et al., 1999). The interaction at the C-3 position appears to also play an important role in EAAT1-expressing C17.2 cells. In this instance, however, the presence of a SO_4^{2-} group, but not acetate, at C-3 appears to be required, as the non-sulfated steroids like PREG, PREGA, EST-hemisuccinate (ESTHem) (at 100μM) were found to have no effect on the uptake of D-aspartate by EAAT1 (Figure 4.5). The non-sulfated steroids, including: PREG and pregnanolone derivatives have also been shown to elicit no effect on NMDA-mediated currents or neurotoxicity in primary rat hippocampal neurons (Weaver et al., 2000).

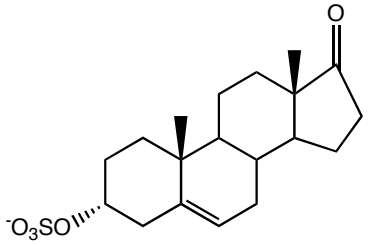
Figure 4.4. Structures of common neurosteroids.



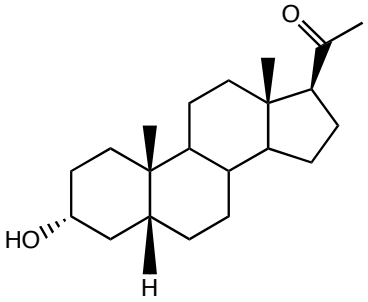
5-Pregnen-3β-ol-20-one (Pregnenolone, PREG)



PREGS

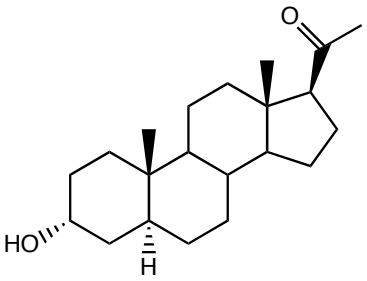


Dehydroepiandrosterone Sulfate (DHEAS)



5β-Pregnan-3α-ol-20-one

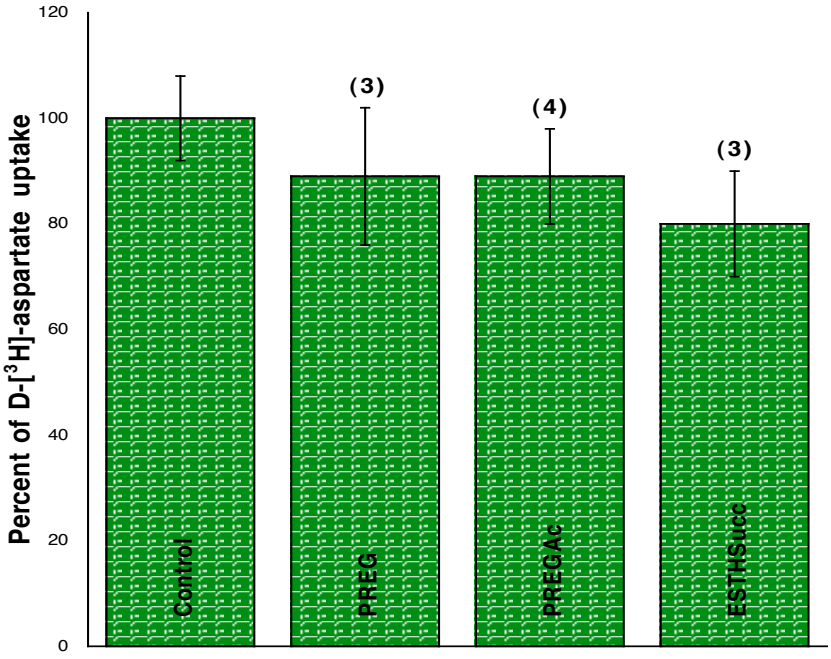
(Pregnanolone, 5β,3α-TH-PROG)



5α-Pregnan-3α-ol-20-one

(Allopregnanolone, 5α,3α-TH-PROG)

Figure 4.5. Effects of non-sulfated steroids on D-[^{3}H]-aspartate uptake by EAAT1 in C17.2 cells.



The transport of D-[^{3}H]-aspartate in C17.2 cells transfected with pAM/CAG-hEAAT1 in the presence and absence of 100µM non-sulfated steroids. The numbers in brackets represent n. The uptake values were found to be statistically non-significant as compared to control.

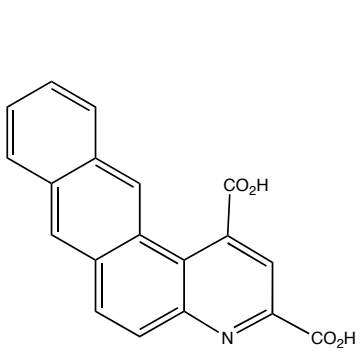
A wide variety of compounds that exhibit some structural characteristics of steroids were also tested for inhibitory activity, including: 5′-adenosine monophosphate (250μM), 5′-guanosine monophosphate (250μM), anandamide (300μM), 5,6-naphthyl quinoline dicarboxylate (5,6-QDC) (250μM) and 3-nitro-L-tyrosine (100μM) (Figure 4.6). None of these compounds showed any effect on the uptake of D-[^{3}H]-aspartate on C17.2 cells expressing EAAT1.

The sulfated steroids exhibit the strongest effects on the uptake by EAAT1

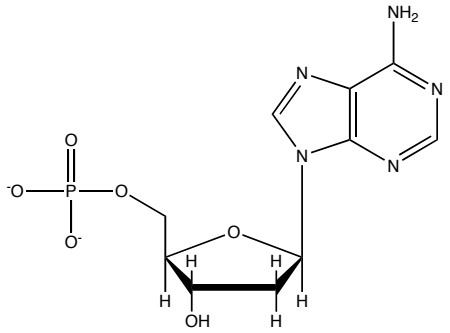
To test if PREGS alters the uptake of D-[^{3}H]-aspartate by other EAATs, C17.2 cells were transfected with EAAT1, EAAT2 or EAAT3. As seen in figure 4.7, uptake into each of the transfected cells was sensitive to the inhibition by L-*trans*-2,4-PDC. In contrast to their effect on EAAT1, PREGS, 3α,5β-TH-PROGS and 3α,5α-TH-PROGS decreased the EAAT2-mediated D-[^{3}H]-aspartate uptake to a small but statistically significant degree: 69±6% (n = 6), 80±4% (n = 5) and 80±5% (n = 6) of control, respectively. In the instance of EAAT3, only PREGS produced a small potentiation that was statistically significant. ESTS at 100μM was ineffective at all the EAAT subtypes tested. Comparison of percent control values clearly suggests the most potent effect of the active steroids is on EAAT1-mediated D-aspartate uptake.

To confirm that the effect of PREGS on EAAT1 is reproducible in a more physiologically relevant system, rat primary astrocytes, which predominantly express EAAT1 (GLAST) (Swanson et al., 1997), were assayed in the presence and absence of PREGS.

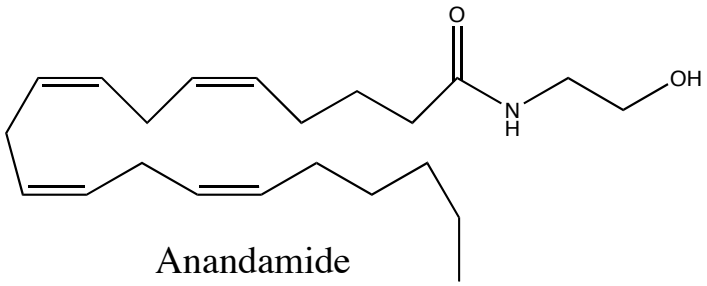
Figure 4.6. Structures of the compounds tested for their activity at EAAT1-mediated uptake in C17.2 cells.



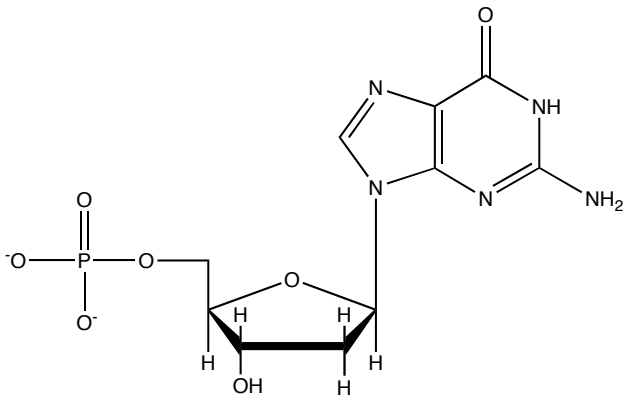
5,6-QDC



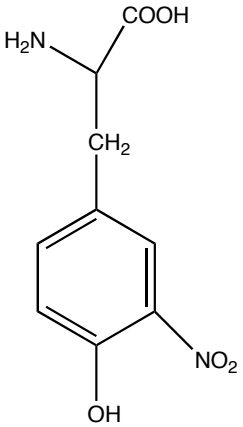
5' AMP



Anandamide

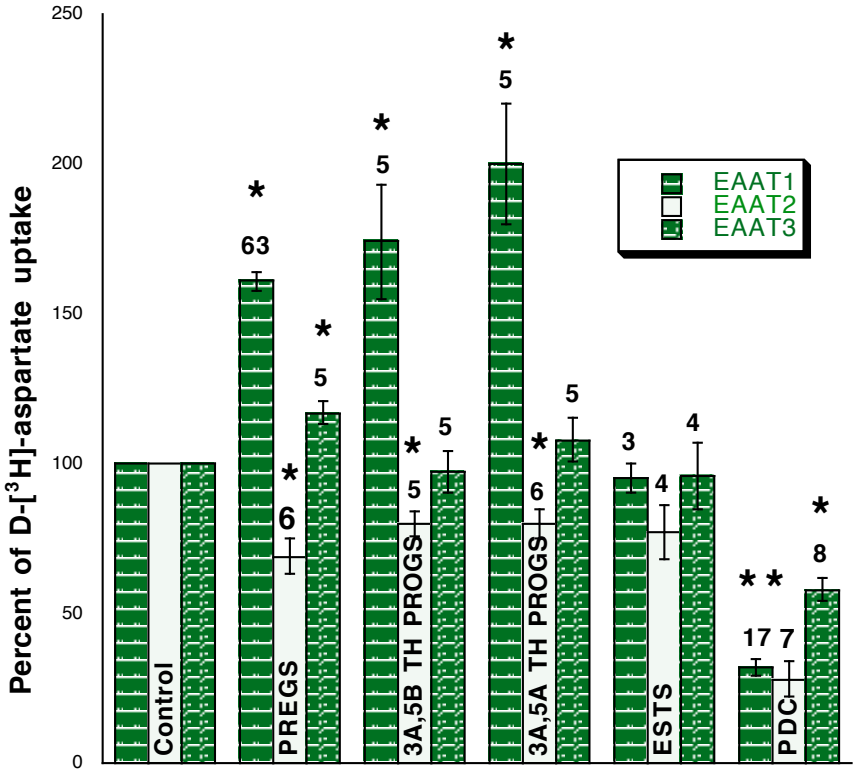


5' GMP



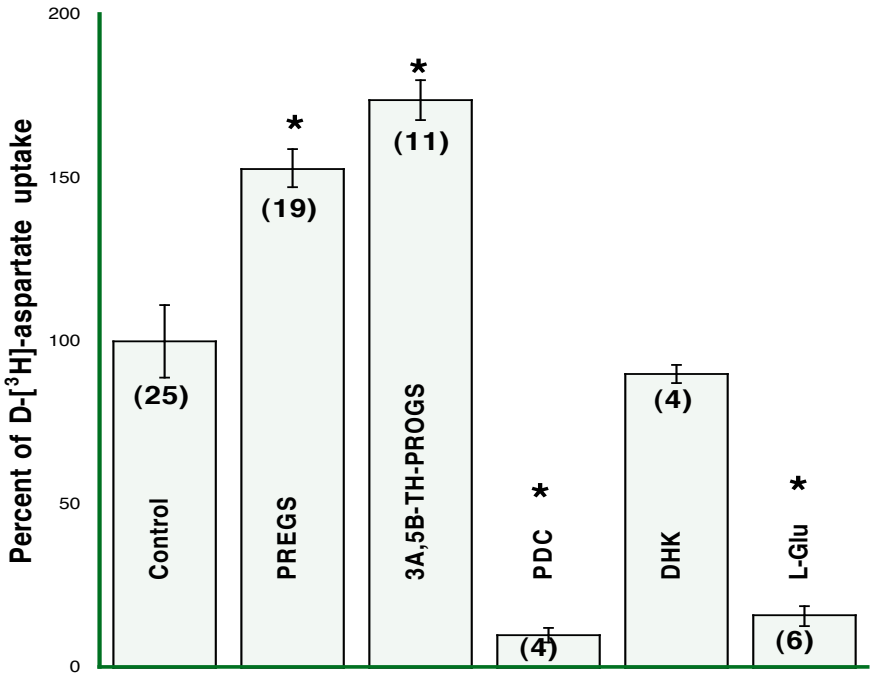
3-Nitro-L-tyrosine

Figure 4.7. Effects of PREGS on the uptake of D-[^{3}H]-aspartate by EAAT1, 2 and 3 in C17.2 cells.



The uptake of D-[^{3}H]-aspartate was measured in C17.2 cells transfected with hEAAT1, hEAAT2 or hEAAT3 in the presence and absence of 100µM sulfated steroids. The D-[^{3}H]-aspartate uptakes were blocked by 100µM L-t-2,4-PDC (non-selective EAAT inhibitor). The numbers in brackets represent n. * ($p < 0.05$) when compared to the corresponding control uptake values.

Figure 4.8. Effect of PREGS on the uptake of D-[^{3}H]-aspartate by primary astrocyte cells in culture (PAC).



The uptake of D-[^{3}H]-aspartate was measured in primary astrocytes. Control uptake for 10μM D-[^{3}H]-aspartate was 165 ± 18 pmol/min/mg protein (mean ± S.E.M., n = 25). The steroids were used at 100μM. The concentrations of inhibitors were 100μM for DL-TBOA and L-glutamate, 250μM for DHK. The data are the mean ± S. E. M. of n individual experiments each performed in duplicate. * $p < 0.05$.

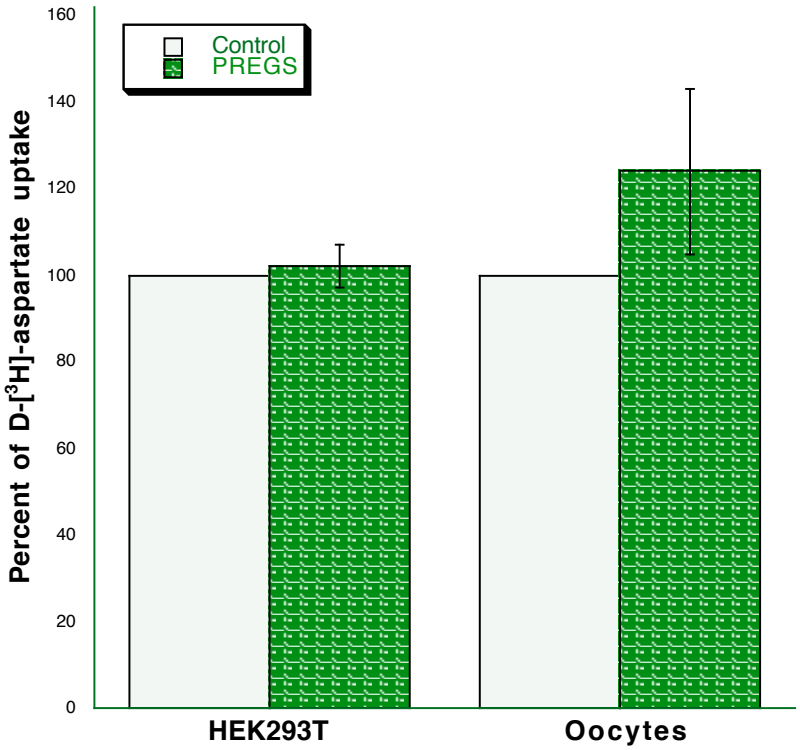
EAAT1 (GLAST) and EAAT2 (GLT-1) are the two subtypes most commonly found on rat astrocytes (Danbolt, 2001). DHK was used as a negative control to exclude any effects attributable to EAAT2. As shown in Figure 4.8, the sodium dependent transport in the primary astrocytes was inhibited by the non-specific EAAT inhibitor, L-*trans*-2,4-PDC but not by the EAAT2-selective blocker, DHK. Consistent with our observations in C17.2 cells, PREGS and 3α, 5β-TH-PROGS, reproducibly stimulated the uptake of D-[^{3}H]-aspartate by the primary astrocytes to 153 ± 6% control (mean ± s.e.m., n = 19) and 174 ± 6% control (mean ± s.e.m., n = 11) . It therefore appears that C17.2 cells may be used as an appropriate mimic of the endogenous system. Two other cell types, HEK293T cells and *Xenopus laevis* oocytes, were also examined (Figure 4.9). Surprisingly, PREGS did not exert any effects on EAAT1-mediated D-aspartate uptake in these cells. This suggests that the stimulatory acitivity is cell-type specific and that additional mechanisms and / or interactions may play a role in the observed effects.

The concentration dependence of the activity of PREGS was then examined in greater detail. The dose-response curves shown in Figures 4.10 and 4.11 reveal that the effect of PREGS is saturable and exhibits the half-maximal (EC_{50}) values of 8±2μM for C17.2 expressing EAAT1 and 4±1μM for the primary astrocytes.

PREGS effect D-aspartate uptake but not the uptake of other common substrates

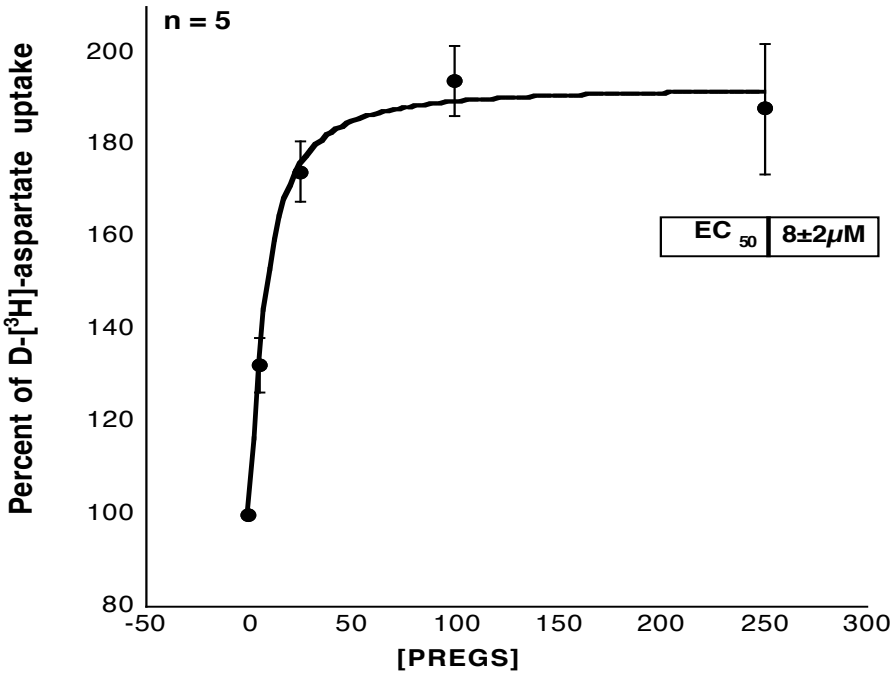
The initial studies on the activity of PREGS on EAAT1 were carried out with D-aspartate as a substrate. This analogue is commonly used in the uptake studies as it negates the metabolic complications associated with the use of either L-glutamate or L-aspartate.

Figure 4.9. Effect of PREGS on the uptake of D-[^{3}H]-aspartate in HEK293T cells and oocytes expressing EAAT1.



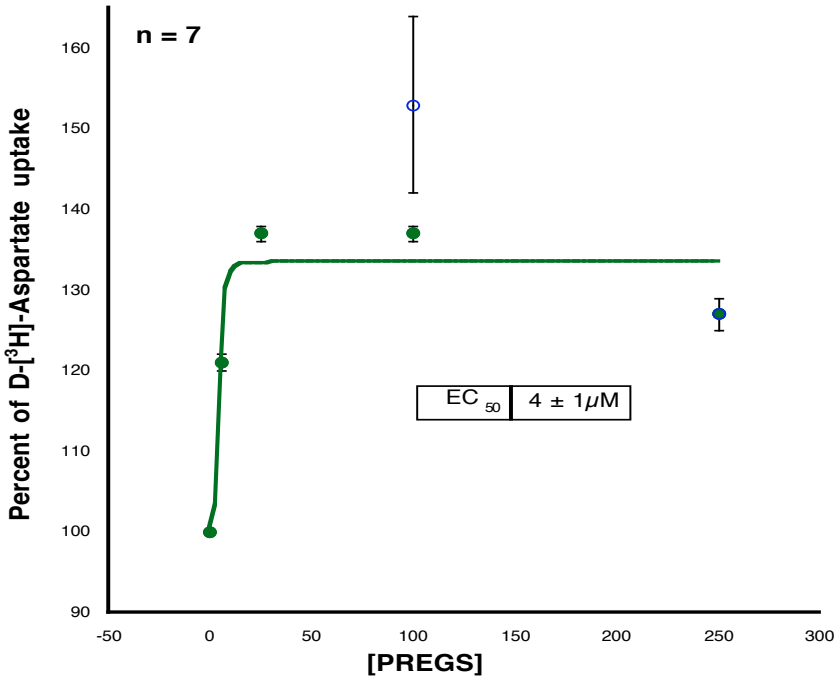
The uptake of D-[^{3}H]-aspartate was measured in HEK293T cells and in oocytes expressing EAAT1. The concentration of D-aspartate used were 10µM for HEK293T cells and 100µM for oocytes. PREGS do not appear to alter the activity of EAAT1 for the uptake of D-[^{3}H]-aspartate in these expression systems. N = 3.

Figure 4.10. Dose-reponse curve showing potentiation of EAAT1-mediated D-[^{3}H]-aspartate uptake by PREGS in C17.2 cells.



Effects of various concentrations of PREGS on the uptake of 10μM D-aspartate uptake in C17.2 cells transfected with pAM/CAG-hEAAT1. EC_{50} values (shown in inset) from the dose-response curves were generated using a four-parameter Hill function. The data were fit to the equation: y = a + b*c/(d^c+x^c), where a = y min, b = range of transition (y max – y min), c = slope, d = EC_{50}.

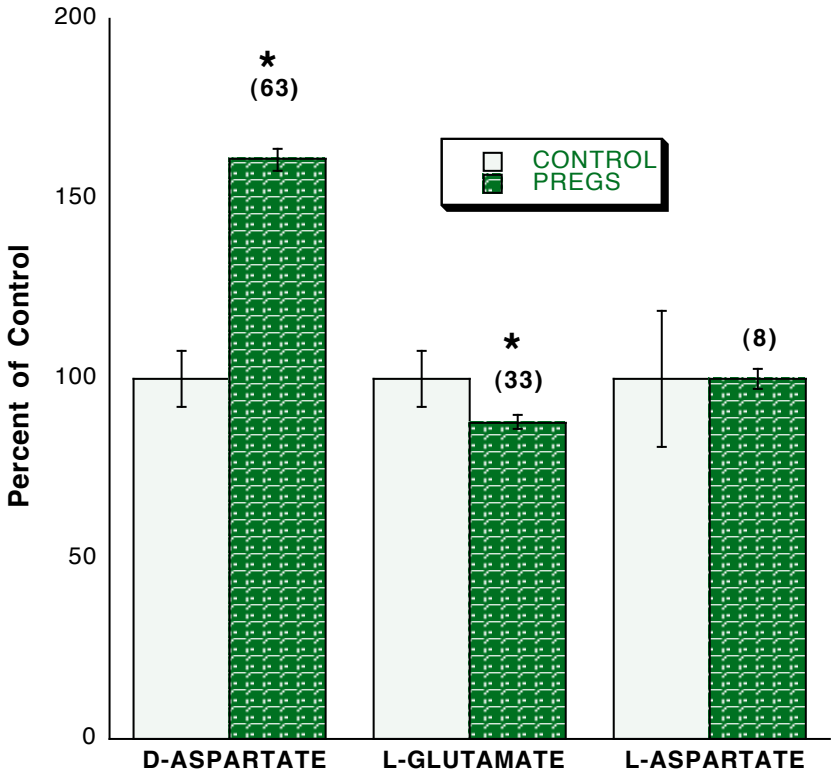
Figure 4.11. Dose-reponse curve showing potentiation of EAAT1-mediated D-[^{3}H]-aspartate uptake in primary astrocytes.



Effects of different concentrations of PREGS on the uptake of 10μM D-aspartate uptake in primary astrocytes. EC_{50} values (shown in inset) from the dose-response curves were generated using a four-parameter Hill function. The data were fit to the equation: y = a + b*c/(d^c+x^c), where a = y min, b = range of transition (y max – y min), c = slope, d = EC_{50}.

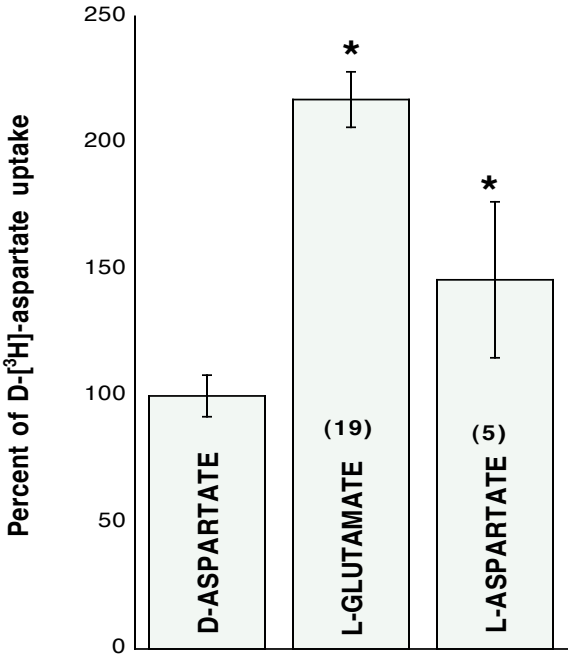
Thus, C17.2 cells expressing EAAT1 were assayed for L-glutamate and L-aspartate uptake in the presence and absence of PREGS. Surprisingly, while PREGS stimulates the uptake of D-[^{3}H]-aspartate, it minimally effected the uptake of L-[^{3}H]-glutamate or L-[^{3}H]-aspartate by EAAT1 (Figure 4.12). The lack of an effect of PREGS on either L-aspartate or L-glutamate uptake prompted us to question if the kinetic properties of the substrates at EAAT1 may influence the observed modulation. To examine this, we first compared the substrate activities of D-[^{3}H]-aspartate, L-[^{3}H]-aspartate and L-[^{3}H]-glutamate under identical conditions (10μM). As shown in Figure 4.13, the accumulation of L-[^{3}H]-glutamate and L-[^{3}H]-aspartate was greater than that observed with uptake of D-[^{3}H]-aspartate potentially resulting from differences in either (or both) K_m and V_{max} values. Thus, about twice as much L-[^{3}H]-glutamate is transported (217±11% of D-aspartate, n = 19) as D-aspartate. The I_{max} value for D-aspartate uptake has been reported to be 0.43% relative to that of glutamate (I_{max} = 1) in oocytes expressing EAAT1 (Arriza et al., 1994). In the same study, the reported K_m values were 48μM and 60μM for L-glutamate and D-aspartate respectively at EAAT1 expressed in COS-1 cells. To further expand this relationship, the concentration dependence with which EAAT1 transports L-glutamate and D-aspartate were examined in the presence and absence of PREGS. The kinetic analysis of L-glutamate and D-aspartate uptake illustrated in figures 4.14 and 4.15 yielded a K_m value for D-aspartate and L-glutamate of 41±3μM and 19±1μM, respectively, in C17.2 cells expressing EAAT1. Interestingly, when expressed in C17.2 cells, the V_{max} values for L-glutamate and D-aspartate were similar. Non-linear analysis of the curves reveal that while PREGS does not significantly alter the V_{max} for either L-glutamate or D-aspartate uptake, it reduces the K_m (21 ± 2μM) for EAAT1-mediated

Figure 4.12. Effect of PREGS on the EAAT1-mediated uptake of D-[^{3}H]-aspartate, L-[^{3}H]-glutamate and L-[^{3}H]-aspartate.



The effect of 100µM PREGS on the uptake of D-[^{3}H]-aspartate, L-[^{3}H]-glutamate and L-[^{3}H]-aspartate, in C17.2 cells transfected with pAM/CAG-hEAAT1. The substrate concentrations were at 10µM. The control uptake rates for the substrates were (in pmol/min/mg): 102 ± 8 (mean ± S.E.M., n =65) for D-[^{3}H]-aspartate, 177 ±15 (n = 39) for L-[^{3}H]-glutamate and 170 ± 33 (n = 7) for L-[^{3}H]-aspartate. The numbers in the brackets represent n for the PREGS treatment. * ($p < 0.05$) compared to the respective control uptake values.

Figure 4.13. Comparisons of uptake of different substrates in C17.2 cells expressing hEAAT1.



The values for the uptake of D-[^{3}H]-aspartate are compared to that of L-[^{3}H]-glutamate or L-[^{3}H]-aspartate. These substrates were used at 10μM concentrations in C17.2 cells transfected with hEAAT1. The numbers in the brackets represent n. * $p < 0.05$.

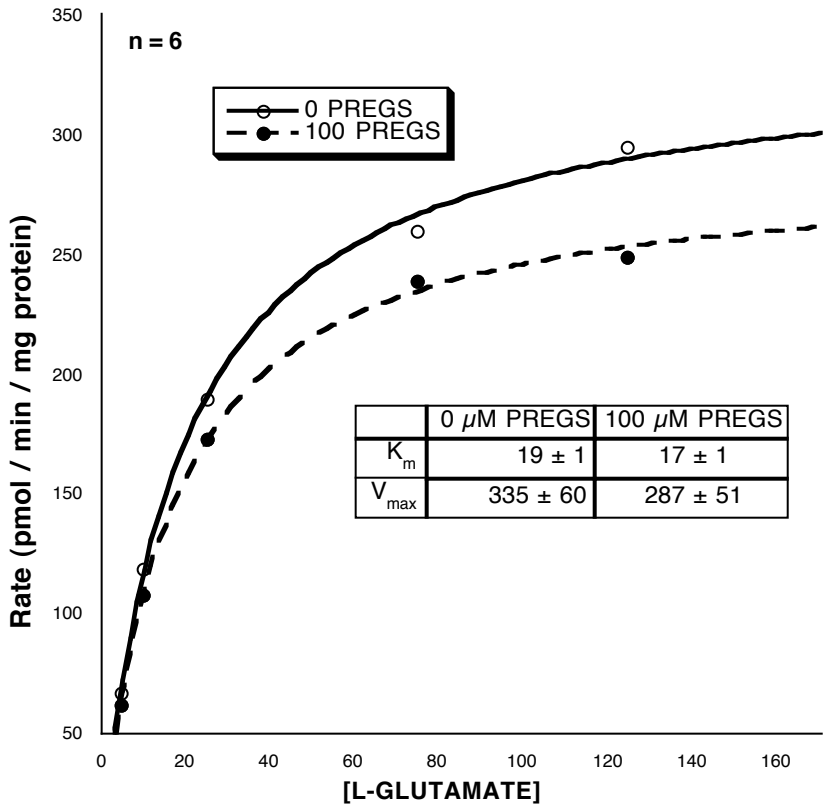
uptake of D-[^{3}H]-aspartate . The effect on K_m suggests potential allosteric modulation of the transporter by PREGS. The K_m for L-glutamate transport did not change in the presence or absence of PREGS.

PREGS appears to increase the uptake of D-[^{3}H]-asparatate by direct interaction with EAAT1

To further characterize the nature of interaction between PREGS and EAAT1-expressing C17.2 cells, pre-incubation studies were undertaken to examine the effects of variables such as exposure time and reversibility. As shown in Table 4.1, the 15′ pre-incubation with PREGS, followed by a 5′ washout, produced virtually no change on the uptake of D-aspartate. These findings suggest that the effects of PREGS are rapid and reversible. Moreover, subsequent treatment with PREGS after the washout produced an equivalent stimulation, further indicating it may be directly interacting with the transporter.

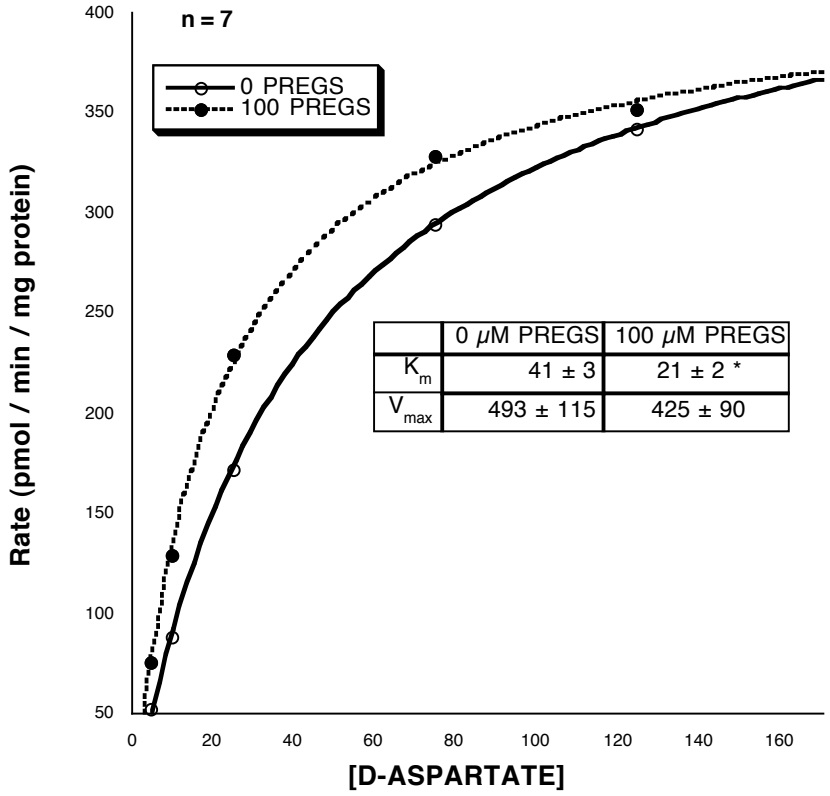
Numerous examples exist that utilize second messenger-mediated mechanisms such as Ca^{2+} ions or nitric oxide molecules to regulate transporter activity (Berry et al., 2005; Duan et al., 1999; Mafra et al., 2002). To investigate if Ca^{2+} contributed to the observed changes in the EAAT1-mediated D-aspartate uptake, assays were conducted in Ca^{2+}-free HBSS uptake buffer containing Ca^{2+} ion chelator EGTA. As shown in Table 4.2, while uptake was depressed a small amount in the Ca^{2+}-free conditions, PREGS still increased the EAAT1-mediated uptake to about 150% of control. Similarly, 60′ preincubation with L-NAME (NO inhibitor) did not prevent the stimulating effect of PREGS on EAAT1 as

Figure 4.14. Michelis-Menton kinetics on the uptake of L-[^{3}H]-glutamate in the presence and absence of PREGS by EAAT1-expressing C17.2 cell.



A representative plot of concentration dependence of L-[^{3}H]-glutamate uptake in C17.2 cells transfected with pAM/CAG-hEAAT1 in the presence and absence of 100μM PREGS. The data were analyzed by non-linear curve-fitting to Michelis-Menton equation, y = m1*x / (m2 + x) The inset shows the V_{max} (m1) and K_m (m2) values (mean ± S.E.M., n = 6).

Figure 4.15. Michelis-Menton kinetics on the uptake of D-[^{3}H]-aspartate in the presence and absence of PREGS by EAAT1-expressing C17.2 cell.



A representative plot of concentration dependence of L-[^{3}H]-aspartate uptake in C17.2 cells transfected with pAM/CAG-hEAAT1 in the presence and absence of 100μM PREGS. The data were analyzed by non-linear curve-fitting to Michelis-Menton equation, y = m1*x / (m2 + x) The inset shows the V_{max} (m1) and K_m (m2) values (mean ± S.E.M., n = 7). * $p < 0.05$.

Table 4.1. Effect of 15-minute PREGS preincubation of D-[^{3}H]-aspartate uptake by C17.2 cells expressing hEAAT1.

Treatment	**Percent of control**
5′ D-[^{3}H]-aspartate uptake	100
5′ D-[^{3}H]-aspartate uptake + PREGS	161 ± 3 (n = 63) *
15’ preincubation with 100μM PREGS + 5′ D-[^{3}H]-aspartate uptake	100
15’ preincubation with 100μM PREGS + 5′ D-[^{3}H]-aspartate uptake + PREGS	143 ± 5 (n = 5) *

C17.2 cell expressing EAAT1 were preincubated with 100μM PREGS for 15′ followed by a 5′ washout. The uptake of D-[^{3}H]-aspartate was subsequently evaluated in the presence or absence of simultaneous application of 100μM PREGS for 5′ followed by the washout. No statistically significant results were obtained when the control uptake rates for D-[^{3}H]-aspartate , in the preincubation condition (106 ± 4 %,) and the non-preincubation conditions, were compared. * ($p < 0.05$) denotes comparison to the respective controls.

Table 4.2. Effect of Ca^{2+}-free conditions on the potentiating effect of PREGS on D-[^{3}H]-aspartate uptake by EAAT1-expressing C17.2 cells.

Treatment	**Percent of control**
5′ D-[^{3}H]-aspartate uptake	100
5′ D-[^{3}H]-aspartate uptake + PREGS	161 ± 3 (n = 63) *
5′ D-[^{3}H]-aspartate uptake (Ca^{2+}-free)	100
5′ D-[^{3}H]-aspartate uptake + PREGS (Ca^{2+}-free)	152 ± 16 (n = 3) *

The uptake of D-[^{3}H]-aspartate was evaluated in normal and Ca^{2+}-free conditions in the presence or absence of simultaneous application of 100μM PREGS for 5′. The control uptake rates for the C17.2 cells in the Ca^{2+}-free conditions were statistically significant (72 ± 4%, n = 3, $p < 0.05$, n = 4) when compared to normal conditions. However, 100μM PREGS stimulated the uptake of D-[^{3}H]-aspartate to the same extent under both conditions. * ($p < 0.05$) denotes comparison to the respective controls.

Table 4.3. Effect of L-NAME preincubation on the potentiating effect of PREGS on D-[^{3}H]-aspartate uptake by EAAT1-expressing C17.2 cells.

Treatment	Percent of control
5′ D-[^{3}H]-aspartate uptake	100
5′ D-[^{3}H]-aspartate uptake + PREGS	161 ± 3 (n = 63) *
5′ D-[^{3}H]-aspartate uptake (L-NAME)	100
5′ D-[^{3}H]-aspartate uptake + PREGS (L-NAME)	205 ± 12 (n = 3) *

C17.2 cells expressing hEAAT1 were preincubated with 100μM L-NAME (NO inhibitor) for 1 hour. After a 5′ washout, the uptake of D-[^{3}H]-aspartate was evaluated in the presence or absence of 100μM PREGS for 5′. The control uptake rates for the C17.2 cells preincubated with L-NAME were statistically non-significant (109 ± 2%, n = 4) when compared to normal conditions. 100μM PREGS stimulated the uptake of D-[^{3}H]-aspartate significantly in both the preincubation and normal conditions. * ($p < 0.05$) denotes comparison to the respective controls.

illustrated in Table 4.3. These results, when combined with rapid and reversible action of PREGS, further suggest a direct allosteric activity at EAAT1.

PREGS appears to preferentially alter the activity of relatively poor substrates. The finding that PREGS increased the uptake of D-[^{3}H]-aspartate, but not L-[^{3}H]-glutamate suggests that however EAAT1 activity is being modulated, it represents a change that does not apply equally to all substrates. This led to the hypothesis that perhaps the action of PREGS on the EAAT1 activity may be dependent upon the individual kinetic properties of the transporter substrates. This would also be consistent with the change in K_m observed for D-aspartate, but not for L-glutamate. Such a possibility is supported by the inhibitor data presented in Tables 4.4 and 4.5. Thus, there was only a minimal change in the level of inhibition produced by DL-β-TBOA on the uptake of L-[^{3}H]-glutamate in the presence of PREGS. However, in agreement with a change in the K_m for D-aspartate, the level of inhibition of D-[^{3}H]-aspartate uptake produced by the same amount of DL-β-TBOA was reduced from 7 ± 1% of control (n = 7) to 20 ± 2% of control (n = 7) when PREGS was present. Taken together, these assays suggest that D-aspartate became a better ligand (i.e., was less sensitive to inhibition by DL-β-TBOA) in the presence of PREGS.

Another way to address this issue is to determine if the potency of weaker inhibitors also increase in the presence of PREGS. To investigate this possibility, a series of substrates were tested for their inhibitor activity against both D-aspartate and L-glutamate in the presence and absence of PREGS. These included L-serine-o-sulfate (L-SOS) (Arriza et

al., 1994; Vandenberg et al., 1998b), 2S,4R-4-methyl glutamate (4MG) (Vandenberg et al., 1997) and L-cysteine (Zerangue and Kavanaugh, 1996b). L-SOS and 4MG have been shown to act as substrates at EAAT1, but not at EAAT2. 4MG and L-SOS have also been reported to be substrates of EAAT1 expressed in oocytes with K_m value of 54μM and 39μM, respectively. The same study yielded a lower K_m value for L-glutamate (K_m = 20μM) (Vandenberg et al., 1997; Vandenberg et al., 1998a). The K_m value for L-cysteine (K_m = 1.8mM) transport at EAAT1 is much higher in comparison to EAAT2 (K_m = 1mM) or EAAT3 (K_m = 191μM) in oocytes (Zerangue and Kavanaugh, 1996b). When these substrates were assayed for their inhibitory activity at EAAT1, each inhibited the uptake of L-[^{3}H]-glutamate to a greater degree in the presence of PREGS (Tables 4.6, 4.8, 4.10). For example, whereas 4MG at 250μM reduced the uptake of 10μM L-[^{3}H]-glutamate to 40 ± 1% of control (mean ± s.e.m., n = 3), it reduced the uptake to a greater degree (21 ± 2% of control, n = 3) in the presence of PREGS. In contrast, when the same compounds were tested against D-[^{3}H]-aspartate uptake, the presence of PREGS produced little change in activity (Tables 4.7, 4.9 and 4.11). Thus, it can be hypothesized that PREGS had no effect on L-glutamate, but enhanced the binding of L-SOS, L-cysteine and 4MG, thereby increasing their inhibitory activities. On the other hand, since PREGS also increased the activity of D-aspartate, all of the compounds were similarly effected and no change in inhibitory activity was observed.

The ability of PREGS to alter the activity of L-cysteine is of particular interest, because it is found endogenously and serves as an important precursor for glutathione (GSH) synthesis (Dringen, 2000). Glutathione has been shown to prevent oxidative injury by

Table 4.4. Effect of DL-β-TBOA on the uptake of L-[^{3}H]-glutamate in the presence and absence of PREGS in C17.2 cell expressing EAAT1.

Treatment	Percent of control
L-[^{3}H]-glutamate uptake	100
L-[^{3}H]-glutamate uptake + DL-TBOA	21 ± 1 (n = 9) *
L-[^{3}H]-glutamate + PREGS	100
L-[^{3}H]-glutamate uptake + PREGS + DL-TBOA	29 ± 2 (n = 8) * †

EAAT1-mediated uptake of 10μM L-glutamate was significantly inhibited by 100μM DL-β-TBOA in the presence and absence of 100μM PREGS (* $p < 0.05$). The neurosteroid, by itself, slightly inhibited the uptake of L-[^{3}H]-glutamate (88 ± 2, n = 33, $p < 0.05$). In the presence of PREGS, the ability of DL-β-TBOA to inhibit the uptake of L-[^{3}H]-glutamate is slightly, but significantly, reduced as compared to the uptake in the presence of DL-β-TBOA alone († $p < 0.05$).

Table 4.5. Effect of DL-β-TBOA on the uptake of D-[^{3}H]-aspartate in the presence and absence of PREGS in C17.2 cell expressing EAAT1.

Treatment	Percent of control
D-[^{3}H]-aspartate uptake	100
D-[^{3}H]-aspartate uptake + DL-TBOA	7 ± 1 (n = 7) *
D-[^{3}H]-aspartate + PREGS	100
D-[^{3}H]-aspartate uptake + PREGS + DL-TBOA	20 ± 2 (n = 7) * †

EAAT1-mediated uptake of 10μM D-glutamate was significantly inhibited by 100μM DL-β-TBOA in the presence and absence of 100μM PREGS (* $p < 0.05$). The neurosteroid, by itself, increased the uptake of D-[^{3}H]-aspartate (161 ± 3, n = 63, $p < 0.05$). In the presence of PREGS, the ability of DL-β-TBOA to inhibit the uptake of D-[^{3}H]-aspartate is significantly reduced as compared to the uptake in the presence of DL-β-TBOA alone († $p < 0.05$).

several mechanisms (Bains and Shaw, 1997). Whether or not EAATs play a significant role in the uptake of L-cysteine as a precursor for GSH remains to be determined. To further investigate the effects of PREGS on L-cysteine activity, we examined whether there were any changes in the kinetic parameters of D-[^{3}H]-aspartate and L-[^{3}H]-glutamate uptake when L-cysteine was included in the presence or absence of PREGS. As discussed earlier, PREGS decreased the K_m for D-aspartate at EAAT1, while the K_m for L-glutamate stayed unaffected (Figures 4.13 and 4.14). When assayed in the presence of 2mM L-cysteine, PREGS reduced the apparent affinity only for L-glutamate (Figures 4.16 and 4.17). Thus, the $K_{m,app}$ for L-glutamate increased from 21±1μM in the presence of 2mM L-CSH to 31±1μM in the presence of both L-CSH and PREGS, while the corresponding V_{max} did not significantly change (248±55pmol/min/mg protein to 181±36pmol/min/mg protein). These effects suggest that L-CSH is more potently competing with L-glutamate in the presence of PREGS than in its absence. In the instance of D-aspartate, L-CSH (2mM) increased the $K_{m,app}$ from 41±3μM to 60±15μM at EAAT1, consistent with competitive inhibition. In contrast to what was observed with L-glutamate, no significant change in these $K_{m,app}$ values were observed in the presence of PREGS. This suggests that D-aspartate and L-CSH may be similarly effected by the action of PREGS on EAAT1.

Boudker *et al*. have identified a lipophilic-binding site in the *Pyrococcus horikoshii* glutamate transporter GLT_{PH} crystal structure (Boudker et al., 2007). They speculate that this may be a potential site of allosteric regulation by lipophilic compounds like arachidonic acid and other PUFAs including docosahexaenoic acid (DHA) and linolenic

Table 4.6. Effect of PREGS on the inhibition of L-[^{3}H]-glutamate by 4-MG.

Treatment	Percent of control
L-[^{3}H]-glutamate uptake	100
L-[^{3}H]-glutamate uptake + 4-MG	40 ± 1 (n = 3) *
L-[^{3}H]-glutamate + PREGS	100
L-[^{3}H]-glutamate uptake + PREGS + 4-MG	21 ± 2 (n = 3) * †

EAAT1-mediated uptake of 10μM L-glutamate was significantly inhibited by 100μM 4-MG in the presence and absence of 100μM PREGS (* $p < 0.05$). The neurosteroid, by itself, slightly inhibited the uptake of L-[^{3}H]-glutamate (88 ± 2, n = 33, $p < 0.05$). In the presence of PREGS, the ability of 4-MG to inhibit the uptake of L-[^{3}H]-glutamate was significantly increased as compared to the uptake in the presence of 4-MG alone († $p < 0.05$).

Table 4.7. Effect of PREGS on the inhibition of D-[^{3}H]-aspartate by 4-MG.

Treatment	Percent of control
D-[^{3}H]-aspartate uptake	100
D-[^{3}H]-aspartate uptake + 4-MG	28 ± 1 (n = 3) *
D-[^{3}H]-aspartate + PREGS	100
D-[^{3}H]-aspartate uptake + PREGS + 4-MG	17 ± 2 (n = 3) * †

EAAT1-mediated uptake of 10μM D-aspartate was significantly inhibited by 100μM 4-MG in the presence and absence of 100μM PREGS (* $p < 0.05$). The neurosteroid, by itself, increased the uptake of D-[^{3}H]-aspartate (161 ± 3, n = 63, $p < 0.05$). In the presence of PREGS, the ability of 4-MG to inhibit the uptake of D-[^{3}H]-aspartate was slightly, but significantly, reduced as compared to the uptake in the presence of 4-MG alone († $p < 0.05$).

Table 4.8. Effect of PREGS on the inhibition of L-[^{3}H]-glutamate by L-SOS in C17.2 cells expressing hEAAT1.

Treatment	Percent of control
L-[^{3}H]-glutamate uptake	100
L-[^{3}H]-glutamate uptake + L-SOS	18 ± 2 (n = 3) *
L-[^{3}H]-glutamate + PREGS	100
L-[^{3}H]-glutamate uptake + PREGS + L-SOS	11 ± 1 (n = 3) * †

250μM L-SOS significantly inhibited the uptake of 10μM L-glutamate in the presence or absence of 100μM PREGS (* $p < 0.05$). The neurosteroid, by itself, slightly inhibited the uptake of L-[^{3}H]-glutamate (88 ± 2, n = 33, $p < 0.05$). L-SOS inhibited the uptake more strongly in the presence of PREGS († $p < 0.05$).

4.9. Effect of PREGS on the inhibition of D-[^{3}H]-aspartate by L-SOS.

Treatment	Percent of control
D-[^{3}H]-aspartate uptake	100
D-[^{3}H]-aspartate uptake + L-SOS	11 ± 2 (n = 3) *
D-[^{3}H]-aspartate + PREGS	100
D-[^{3}H]-aspartate uptake + PREGS + L-SOS	8 ± 2 (n = 3) *

PREGS by itself has the ability to potentiate the activity of EAAT1 to transport D-[^{3}H]-aspartate (161 ± 3, n = 63, $p < 0.05$). The uptake of 10μM D-aspartate was significantly inhibited by 250μM L-SOS in the presence and absence of 100μM PREGS (* $p < 0.05$). However, PREGS exerted no effect on the ability of L-SOS to inhibit the uptake of D-[^{3}H]-aspartate.

Table 4.10. Effect of PREGS on the inhibition of L-[^{3}H]-glutamate by L-cysteine (L-CSH) in C17.2 cells expressing hEAAT1.

Treatment	Percent of control
L-[^{3}H]-glutamate uptake	100
L-[^{3}H]-glutamate uptake + L-cysteine	97 ± 4 (n = 3)
L-[^{3}H]-glutamate + PREGS	100
L-[^{3}H]-glutamate uptake + PREGS + L-cysteine	74 ± 2 (n = 3) * †

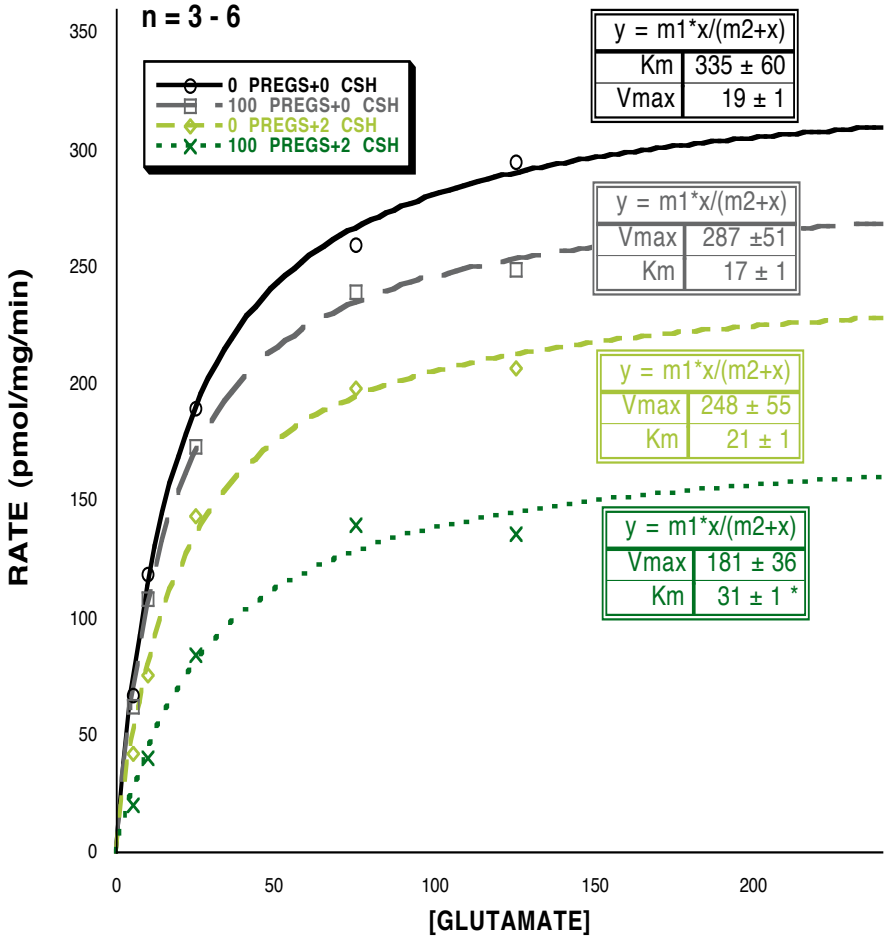
The endogenous substrate, L-cysteine at 1mM, failed to inhibit the uptake of L-[^{3}H]-glutamate. PREGS exhibited a slight inhibitory effect on the ability of EAAT1 to translocated L-glutamate (88 ± .2 % of control, n = 33, $p < 0.05$). However, the same concentration of L-cysteine was able to inhibit the uptake in the presence of 100μM PREGS. This inhibition was statistically significant as compared to the uptake in the presence of L-cysteine alone (†, $p < 0.05$) or PREGS alone (*, $p < 0.05$).

Table 4.11. Effect of PREGS on the inhibition of D-[^{3}H]-aspartate by L-cysteine (L-CSH) in C17.2 cells transfected with hEAAT1.

Treatment	Percent of control
D-[^{3}H]-aspartate uptake	100
D-[^{3}H]-aspartate uptake + L-cysteine	89 ± 2 (n = 3) *
D-[^{3}H]-aspartate + PREGS	100
D-[^{3}H]-aspartate uptake + PREGS + L-cysteine	69 ± 3 (n = 3) *†

PREGS by itself has the ability to potentiate the ability of EAAT1-mediated uptake of D-[^{3}H]-aspartate (161 ± 3, n = 63, $p < 0.05$). The uptake of 10μM D-aspartate was significantly inhibited by 1mM L-cysteine in the presence and absence of 100μM PREGS as compared to control (* $p < 0.05$). The inhibition of D-aspartate uptake by L-cysteine was significantly increased as compared to the transport of D-aspartate in the presence of L-cysteine alone.

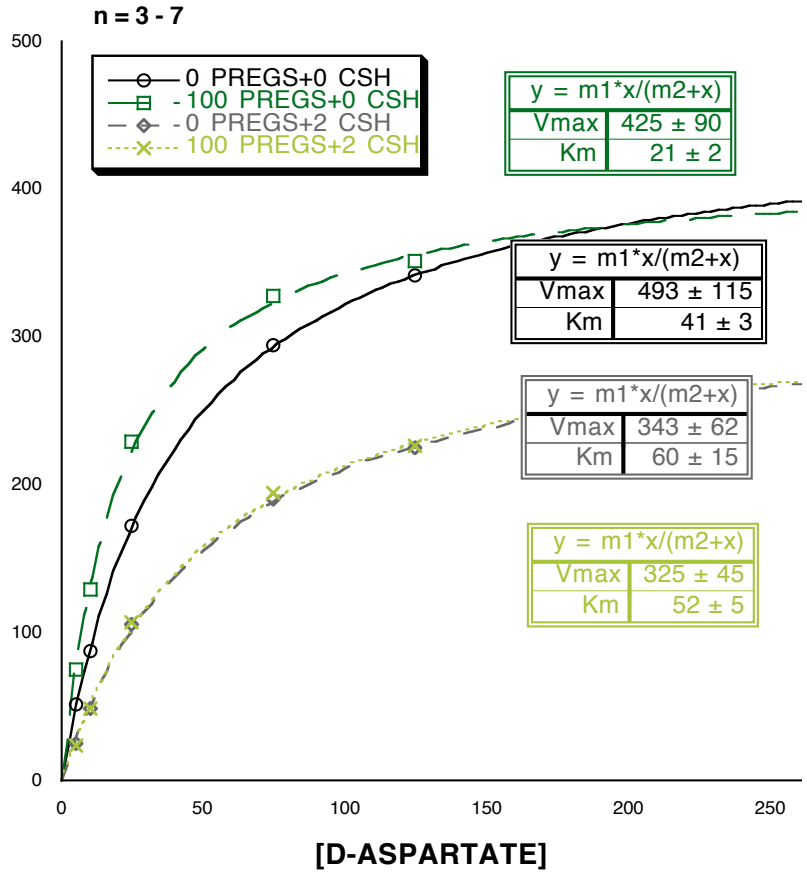
Figure 4.16. Michelis-Menton kinetics on L-[^{3}H]-glutamate uptake by EAAT1 in the presence of PREGS and L-cysteine (L-CSH)



A representative plot of concentration dependence of L-[^{3}H]-glutamate uptake in C17.2 cells transfected with pAM/CAG-hEAAT1. The data were analyzed by non-linear curve-fitting to Michelis-Menton equation, y = m1*x / (m2 + x) The inset shows the V_{max} (m1) and K_m (m2) values (mean ± S.E.M.). 100µM PREGS or 2mM L-cysteine did not alter

either the K_m or the V_{max} of L-[^{3}H]-glutamate uptake in these cells. However, the K_m significantly reduced (* $p < 0.05$) from 17 ± 1μM to 31 ± 1μM when the L-glutamate uptake was measured in the presence of both, PREGS and L-cysteine. The insets, showing the K_m and V_{max} values, are placed next to the respective curve.

Figure 4.17. Michelis-Menton kinetics on D-[^{3}H]-Aspartate uptake by EAAT1 in the presence of PREGS and L-cysteine (L-CSH).



A representative plot of concentration dependence of D-[^{3}H]-aspartate uptake in C17.2 cells transfected with pAM/CAG-hEAAT1. The data were analyzed by non-linear curve-fitting to Michelis-Menton equation, y = m1*x / (m2 + x) The insets, showing the V_{max} (m1) and K_m (m2) values (mean ± S.E.M.), are placed next to the respective curves. 100µM PREGS significantly decreased (* $p < 0.05$), while 2mM L-cysteine significantly increased († $p < 0.05$) the K_m for the D-[^{3}H]-aspartate uptake in these cells. The presence

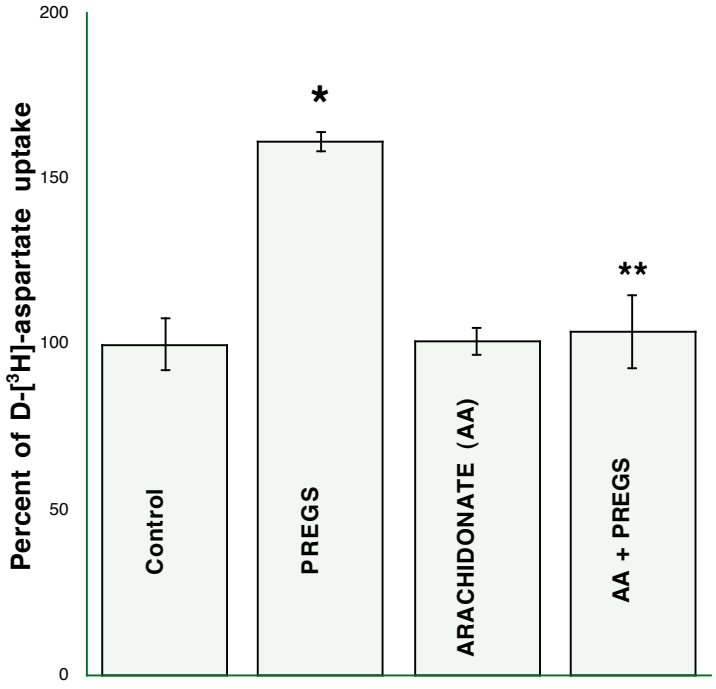
of PREGS significantly decreased the K_m (# $p < 0.05$) as compared to the control. No changes were observed in the ability of L-cysteine to inhibit the uptake of D-aspartate in the presence of PREGS.

acid (Fairman et al., 1998; Zerangue et al., 1995; Tzingounis et al., 1998). Arachidonic acid inhibits EAAT1, stimulates EAAT2 and affects the channel properties of EAAT4 (Fairman et al., 1998; Zerangue et al., 1995; Tzingounis et al., 1998). The possibility that neuroactive steroids might interact with EAAT1 through the similar or related site as arachidonic acid was tested by using PREGS in the presence and absence of this known modulator. Whereas arachidonic acid by itself did not show any effect on the uptake of D-[^{3}H]-aspartate by EAAT1 in C17.2 cells, it did prevent PREGS from stimulating the uptake of D-[^{3}H]-aspartate when coadministered (Figure 4.18). One possible interpretation of these results is that arachidonic acid and PREGS may compete for the same modulatory site on EAAT1.

Chapter 4: Discussion

EAATs are highly regulated proteins. Their modulation has been shown to be mediated by different mechanisms, including: changes in transcription, translation (Rothstein et al., 2005; Tian et al., 2007; Su et al., 2003; Figiel et al., 2003; Schlüter et al., 2002; O'Shea et al., 2006; Thorlin et al., 1998), trafficking (Gonzalez and Robinson, 2004; Hughes et al., 2004) and allosteric regulation (Vandenberg et al., 2004). In the present work, we have identified a putative modulatory site on glutamate transporters that can differentially regulate the function of individual EAAT subtypes. More specifically, we have identified a series of compounds that alter the transport activity of EAAT1, but not EAAT2 or EAAT3. The fact that this observed change in activity was produced by neurosteroids raises questions not only related to the regulatory mechanisms itself, but also to the

Figure 4.18. Effect of co-presence of PREGS and arachidonic acid on EAAT1-mediated D-[^{3}H]-aspartate uptake in C17.2 cells.



The uptake of D-[^{3}H]-aspartate by EAAT1 is significantly increased in the presence of 100μM PREGS in C17.2 cells (161 ± 3% of control, n = 63, * $p < 0.05$). This increase in uptake was blocked by the treatment with 300μM arachidonic acid. ** $p < 0.05$, as compared to the uptake in the presence of PREGS alone.

possible role of neurosteroid in modulating glutamate transport. With respect to the modulatory activity itself, much of the evidence indicating that the effects observed with the neurosteroids reflect a genuine regulatory mechanism is linked to the issue of specificity. Thus, a specificity of action was observed among the different EAAT subtypes, neurosteroids and EAAT substrates.

The neurosteroid, PREGS, was found to significantly increase the uptake of D-[^{3}H]-aspartate in C17.2 cells transfected with hEAAT1. It slightly inhibited the uptake D-[^{3}H]-aspartate by EAAT2 while minimally affecting the activity of uptake by EAAT3. The differential modulation of EAATs has also been reported to occur through variety of other mechanisms. Polyunsaturated fatty acids (PUFAs), as well as Zn^{2+}, have previously been shown to produce differing effects on the EAAT activities by allosteric mechanisms (Vandenberg et al., 1998a; Vandenberg et al., 2004). For example, Zn^{2+} has been shown to bind and modulate EAATs expressed in oocytes (Mitrovic et al., 2001). Whereas it has no effects on EAAT2 and EAAT3, Zn^{2+} was shown to modulate the activity of EAAT1 and EAAT4. It was reported to reduce the glutamate transport by EAAT1 and selectively inhibit the chloride conductance of EAAT4 without affecting substrate currents.

Evidence that the EAATs are individually regulated also comes from studies focusing on protein kinase C (PKC). Thus, PKC activation by phorbol ester, phorbol 12-myristate 13-acetate (PMA) has been shown to decrease the cell surface expression of GLT-1 (EAAT2) in C6 glioma cells and rat astrocyte-neuron co-cultures (Kalandadze et al.,

2002; Zhau and Sutherland, 2004). In contrast, the activation of PKC has been reported to cause a significant increase in both, the cell surface expression and uptake of L-[^{3}H]-glutamate, in C6 glioma cells and astrocyte-neuron cocultures (Davis et al., 1998; Gonzalez et al., 2003; Gonzalez et al., 2002). Importantly, the different PKC subtypes appear to regulate these transporters by distinct mechanisms. Whereas, PKC_α appears to mediate the redistribution of EAAC1 to the cell membrane by direct interaction of with EAAC-1 protein (Gonzalez et al., 2003), PKC_ε seem to increase the intrinsic activity of this transporter in C6 glioma cells (Gonzalez et al., 2002). In the instance of GLAST (EAAT1), acute treatment with PMA has been shown to cause ~20% increase in transport by increasing catalytic efficiency/turnover number of GLAST (Susarla et al., 2004). Furthermore, EAATs are known to be differentially regulated at the level of protein synthesis. Several chemical entities, including corticosterone and retinol, have been identified that stimulate the translation of EAAT2 transcript (Tian et al., 2007). Additionally, several treatments have been shown to increase the expression of *glt1* gene. These include: treatment with β-lactam antibiotic, ceftriaxone (Rothstein et al., 2005), certain extracellular factors, such as EGF (Zelenaia et al., 2000), injury-induced growth factors (TGFα, FGF-2 and PDGF) (Figiel et al., 2003; Schlüter et al., 2002), and lipopolysaccharide (O'Shea et al., 2006).

The effects of PREGS on the uptake activity of glutamate transporters were reproducibly observed with only a sub-group of structurally related sulfated steroids. Thus, pregnanolone sulfate (3α,5β-TH PROGS) and allopregnanolone sulfate (3α,5β-TH PROGS) also stimulated the uptake of D-[^{3}H]-aspartate by EAAT1. These compounds

also exhibited an inhibitory effect on EAAT2 and no significant effect on EAAT3-mediated transport. DHEAS only slightly potentiated the uptake of D-[^{3}H]-aspartate by EAAT1 whereas ESTS had no effect. The non-sulfated steroids, including: PREG, PREGA and ESTH, do not appear to elicit any effect on the uptake activity of EAAT1. These results suggest that the acyl side chain present on C-17 of PREGS, 3α, 5α-TH-PROGS and 3α, 5β-TH-PROGS and the presence of C-3 sulfate group may be important for interaction with the transporter. The introduction of sulfate group at C-3 of neurosteroids has been shown to have dramatic effects on their activity at $GABA_A$ and NMDA receptors. Whereas, the non-sulfated neurosteroids, 3α, 5α-TH-PROG and 3α, 5β-TH-PROG, are reported to activate the GABA-mediated currents at nanomolar concentrations (Belelli and Lambert, 2005), their sulfated counterparts are chiefly negative allosteric modulators of $GABA_A$ receptors at micromolar concentrations (Gibbs et al., 2006; Park-Chung et al., 1999). In the instance of NMDAR, only the sulfated, but not non-sulfated, steroids were reported to be active in micromolar range (Park-Chung et al., 1997; Gibbs et al., 2006). The C-17 side chain and C-3 sulfate group have been identified to be important for the binding of other steroids (Harrison et al., 1987) to a variety of proteins by means of hydrogen bonding with polar or charged residues within the protein, e.g., estrogen binding to estrogen receptors (Brzozowski et al., 1997), 5α-dihydrotestosterone (5α DHT) binding to sex-hormone-binding globulin (SHBG) protein (Grishkovshaya et al., 2000) and pregnanolone binding to the $GABA_A$ receptors (Hosie et al., 2006).

Significantly, the effect of PREGS on EAAT1-mediated activity was also observed in rat primary astrocytes that endogenously express EAAT1 (GLAST) (Swanson et al., 1997). EAAT1 is differentially expressed throughout the mammalian brain (Lehre and Danbolt, 1998; Rauen, 2000; Furness and Lehre, 1997; Berger and Hediger, 2000). It has been shown to be primarily present on astrocytes and is the major glutamate transporter in the cerebellum, the inner ear, the circumventricular organs and the retina. The disruption of GLAST transport activity, either by genetic deletion or antisense oligonucleotide techniques, has been shown to alter the functions of these systems (Maragakis and Rothstein, 2004). The GLAST knockout mice have been reported to exhibit motor incoordination and increased susceptibility to cerebellar cold-induced injury (Watase et al., 1998). Moreover, the intraventricular administration of GLAST antisense oligonucleotides before the induction of ischemia has been shown to result in an exacerbation of neuronal injury (Tao et al., 2001). Consistently, the knockdown of GLAST by the injection of antisense oligonucletide in mice brain has been demonstrated to produce elevated extracellular glutamate levels, neurodegeneration, and a progressive motor deficit (paralysis) (Rothstein et al., 1996). Similarly, the GLAST-knockout mice have shown to increase susceptibility to seizures (Watanabe et al., 1999), exacerbate hearing loss caused by the increased accumulation of glutamate after acoustic-overstimulation (Hakuba et al., 2000) and increase ischemia-induced damage to the retina (Harada et al., 1998). Thus, the modulation of EAAT1 may play a critical role in the regulation of several important functions ascribed to these systems such as coordination and planning of movements, learning motor tasks, balance, hearing and vision (Ghez and Thach, 2000; Hudspeth, 2000; Tessier-Lavigne, 2000) .

While PREGS did increase EAAT1 activity in the C17.2 cells and primary astrocytes, it did not stimulate the activity of EAAT1 in every cell system examined. Thus, PREGS failed to alter the uptake of D-[^{3}H]-aspartate in HEK293T cells or oocytes expressing EAAT1. Inherent differences in the lipid composition among cell membranes, as well as variations in the presence or absence of interacting proteins (London and Brown, 2000; Simons and Ikonen, 1997) may contribute to different functional responses to PREGS and related neuroactive steroids (Moore, 2001). Consistent with such idea, the influence of cell-type on the modulation of EAAT3 by PKC is well documented. In contrast to the increased expression of EAAT3 in C6 cells and neuron-astrocytes co-cultures, the activation of PKC by PMA treatment has been reported to downregulate the EAAC1 (EAAT3) surface expression in oocytes (Trotti et al., 2001) and human astrocytoma cell line U373 (Dunlop et al., 1999).

Surprisingly, the effects of PREGS could not be extended to the uptake of well-recognized substrates, L-glutamate and L-aspartate. This led us to the idea that neuroactive steroids may be acting at this site in a way that alters the transport properties of poor substrates, but has little or no effect on the uptake of good substrates. Previous studies have demonstrated that EAAT1 transports L-glutamate and L-aspartate with greater I_{max} values than that for D-aspartate (Arriza et al., 1994). In this study using C17.2 cells expressing EAAT1, while the V_{max} for both L-glutamate and D-aspartate were similar, the K_m values differed significantly. Consistent with these results, the uptake rates for both L-[^{3}H]-glutamate and L-[^{3}H]-aspartate were higher that those observed for D-[^{3}H]-aspartate in our experiments. Significantly, the increased transport of D-[^{3}H]-

aspartate uptake observed in the presence of PREGS could be ascribed to a significant decrease in the K_m value. Importantly, to make sure the effect of PREGS was not limited to only D-aspartate, we tested a number of other substrates. Certain other ligands with substrate activity at EAAT1 appeared to be similarly effected when tested indirectly for a change in their ability to inhibit the uptake of L-[^{3}H]-glutamate or D-[^{3}H]-aspartate. Thus, PREGS increased the level of inhibition of L-[^{3}H]-glutamate uptake produced by 4MG, L-SOS and L-cysteine. However, it failed to alter the level of inhibition produced by these same substrates on the uptake of D-[^{3}H]-aspartate. Furthermore, the presence of PREGS caused a significant decrease in the inhibition produced by a non-transportable inhibitor, DL-β-TBOA, on the uptake of D-[^{3}H]-aspartate by EAAT1. Consistent with the inability of PREGS to alter the inhibitory activities of L-SOS, 4MG or L-cysteine when assessed against D-aspartate, the neurosteroid also failed to alter the $K_{m,app}$ by L-cysteine in a more detailed kinetic studies with D-[^{3}H]-aspartate. In contrast, the presence of PREGS caused an increase in the $K_{m,app}$ value for L-[^{3}H]-glutamate uptake when L-cysteine was used as an inhibitor. This suggests that L-cysteine becomes a better competitive inhibitor of L-glutamate uptake by EAAT1 in the presence of PREGS.

Consecutive preincubation with PREGS followed by a 5´ washout did not effect the D-aspartate uptake in C17.2 cells expressing EAAT1. Only the simultaneous application of D-[^{3}H]-aspartate and PREGS could produce the observed increase in uptake. Taken together, these findings support the hypothesis that PREGS interacts directly at a modulatory site on glutamate transporter protein, EAAT1. As PREGS did not alter the uptake of L-glutamate, it is possible that the neurosteroid may be only partially active at

this site, whereas a full "agonist" would be expected to increase the uptake of the endogenous substrate. In this respect, the neurosteroid may have led to a discovery of a regulatory site, for which the true physiological ligands have yet to be identified.

However, the fact that PREGS is a neurosteroid and is found endogenously lends another level of interest to the observed effects. PREGS and DHEAS are among the most abundant neurosteroids found in mammalian brain, a finding consistent with neuromodulation. In mammals, glial cells are considered to be a major site of neurosteroid formation and metabolism in the brain. Both oligodendrocytes and astrocytes have been identified as primary site for the synthesis of PREGS (Le Goascogne et al., 2007; Jung-Testas et al., 1989; Baulieu, 1997; Compagnone and Mellon, 2000). However, newer evidence suggests that neuronal cells may also participate in the biosynthesis of neurosteroids from cholesterol. For example, Purkinje cell, a cerebellar neuron, is an active neurosteroidogenic cell, which possesses requisite enzymes to produce PREG, PREGS and progesterone in several vertebrate species (Tsutsui et al., 2003). Considering that EAAT1 is the major glutamate transporter in the cerebellar Bergmann glia, the neurosteroidogenesis in the Purkinje cells is consistent with the idea that neurosteroids may modulate excitatory neurotransmission in the cerebellum through an action on EAAT1.

It has been reported that the anterior rat brain contains about 38ng (~80nM) and 16ng (~35nM) of PREG and PREGS, respectively, per gram of tissue (Corpechot et al., 1983). This level is much higher than the 1ng/g concentrations reported to be present in plasma.

Similarly, DHEAS concentrations in the brain have been reported to be much higher in anterior and posterior rat brain (2ng/g and 5ng/g) as compared to plasma (0.2ng/g) (Corpechot et al., 1981). In the aged human brain, the concentrations of PREGS and DHEAS were reported to be about 2ng/g and 13ng/g tissue (Liere et al., 2004). It is readily acknowledged that these reported concentrations of PREGS are much lower than the micromolar concentrations found to be effective in the present study. Thus, the physiological significance of the putative modulatory activity on the EAATs is still unclear. The micromolar concentrations used in this study are, however, within the same range at which the sulfated steroids that have been shown to alter the activities of other targets, such as NMDA and $GABA_A$ receptors (Wu et al., 1991; Majewska, 1992; Gibbs et al., 2006). An issue that has made the concentration-dependence of all of these effects difficult to interpret, is the inherent problem of quantifying neurosteroids in the extracellular microenvironment. Thus, the concentration of the neurosteroid may reach the micromolar levels needed to act at the $GABA_A$, NMDA and possibly the EAATs, if synthesized or released in local environment surrounding the protein targets.

Interestingly, the effects of PREGS on EAAT1 and 2 are opposite to the effects reported with arachidonic acid (Zerangue et al., 1995; Fairman et al., 1998; Tzingounis et al., 1998; Poulsen and Vandenberg, 2001). Micromolar levels of arachidonic acid were found to inhibit glutamate uptake mediated by EAAT1 by reducing the maximal transport rate approximately 30%. In contrast, arachidonic acid appears to increase the EAAT2 apparent affinity for glutamate more than 2-fold in oocytes and HEK293 cells (Zerangue et al., 1995). There is no change in the uptake of glutamate by EAAT4 in the presence of

arachidonic acid, although it does influence the proton current associated with this transporter (Fairman et al., 1998; Tzingounis et al., 1998; Poulsen and Vandenberg, 2001). In our experiments, arachidonic acid by itself did not produce any effect on the uptake of D-aspartate in C17.2 cells expressing EAAT1. However, its simultaneous application prevented PREGS from stimulating the uptake of ^{3}H-D-aspartate. These results raise the possibility that arachidonic acid and PREGS may be acting at the same site. The most straightforward interpretation would suggest that PREGS is acting as an agonist to stimulate uptake, while arachidonic acid is acting as an antagonist to block its effect. Such an interpretation, however, does not take into account previous reports that arachidonic acid acts as an allosteric modulator to inhibit the uptake by EAAT1. In light of this, one could propose an alternate hypothesis in which arachidonic acid is an agonist acting to inhibit uptake, while PREGS acts as an antagonist that blocks the effect of arachidonic acid and restores EAAT1 activity to increased levels. In turn, this would suggest that arachidonic acid may be acting endogenously in the C17.2 cells and primary astrocyte cultures to constitutively reduce EAAT1 activity. In such a scenario, the addition of PREGS as an antagonist would produce the observed increase in activity. Similarly, if the arachidonic acid is already present, it would provide an explanation as to why the added arachidonic acid had no effects by itself. Unfortunately, neither of the mechanisms provides an explanation as to why the PREGS did not alter the uptake of L-glutamate or L-aspartate. Further studies will take an electrophysiological approach and see if the effects of arachidonic acid on glutamate uptake can be influenced by PREGS. Initial studies in this proposal tested PREGS by itself on oocytes expressing EAAT1 and

found no change in activity. It remains to be seen if PREGS would block the inhibitory activity of arachidonic acid in this same paradigm.

Lastly, in a recent article on the crystal structure of substrate-bound GLT_{PH}, an archeael EAAT homologue *Pyrococcus horikoshii* (Boudker et al., 2007), a lipophilic binding site has been predicted. Curiously, this binding site was apparent in the substrate-bound state of the transporter but not in non-transportable inhibitor bound state. Given our results of PREGS on substrates, it is tempting to speculate this might be its site of action on the EAAT1 protein.

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