

Research Overview

Medicinal Chemistry and Molecular Pharmacology of GABA Receptors and Glutamate Transporters—Complementary Structure–Activity Relationships

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Strategy, Management and Health Policy				
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ABSTRACT GABA and glutamate are the major inhibitory and excitatory neurotransmitters, respectively, in the human brain. Many GABA and glutamate receptors and transporters are key protein targets for drug development, and many known CNS drugs act on these targets. There have been a substantial number of traditional studies of structure–activity relationships in this area. The advent of modern molecular biology using recombinant DNA technology enables studies of structure–activity relationships to be carried out on these protein targets, thus complementing structure–activity relationships for the ligands interacting with these targets. This is illustrated with examples from our investigations of subtypes of GABA_C receptors and glutamate transporters using both native and chimeric proteins of known amino acid sequence expressed in *Xenopus* oocytes. Studies of such complementary structure–activity relationships involving structural variations of both the ligands and their targets will play important roles in drug development. Such studies are vital to the development of drugs that interact selectively with particular native and mutant protein receptor/transporter subtypes. Drug Dev. Res. 46:255–260, 1999. © 1999 Wiley-Liss, Inc.

Key words: GABA; glutamate; CNS drugs; receptors; transporters

INTRODUCTION

The simple amino acids, GABA and glutamate, are the major inhibitory and excitatory neurotransmitters, respectively, in the human brain. Most, if not all, nerve cells in the brain have membrane receptors for extracellular GABA and glutamate. Highly specific transporters maintain low extracellular concentrations of these amino acids. These transporters also serve to remove synaptically released GABA and glutamate from the synaptic environment, thus rapidly terminating their synaptic actions. A wide range of CNS-active agents is known to act selectively on aspects of the GABA and glutamate transmitter systems, e.g., anticonvulsants, barbiturates, benzodiazepines, ethanol, steroids, and neurotoxins.

GABA and glutamate are flexible molecules that can adopt a variety of low-energy conformations capable of interacting with different receptors and transporters.

Studies on a range of conformationally restricted analogs of GABA and glutamate resulted in an awareness of the existence of a variety of subtypes of receptors and transporters for these amino acid neurotransmitters. Modern molecular biology has shown that many more subtypes exist than were predicted from pharmacological studies.

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A large number of receptors and transporters for GABA and glutamate have been cloned. These form families of structurally related membrane proteins with a high degree of amino acid sequence conservation.

The use of recombinant DNA technology to express receptors and transporters of known sequences in cells that do not normally express these proteins enables precise functional structure–activity relationships to be studied both on the ligands that interact with these proteins and on the proteins themselves. This approach to developing complementary structure–activity relationships of ligands and their targets will be illustrated by our work on GABA receptors and glutamate transporters.

GABA RECEPTOR FAMILIES

Three families of GABA receptors have been cloned thus far. GABA_A and GABA_C receptors are transmitter-gated ion channel receptors thought to be made up of five protein subunits with an integral chloride ion channel being formed in the middle of these subunits (Fig. 1). GABA_A receptors appear to be relatively complex, with at least three different proteins constituting the functional receptors, i.e., they are heteromeric [Johnston, 1996a]. On the other hand, GABA_C receptors appear to be relatively simple, being made up of five identical protein macromolecules, i.e., they are homomeric [Johnston, 1996b].

The functional properties of these receptors may be studied by expression of the clones in cells that do not normally express GABA receptors; the most common cells used in such studies are oocytes from the South African frog *Xenopus laevis*. Injection of cRNAs coding for the different receptor proteins into the oocytes results in the expression 2–7 days later of functional GABA receptors, which can be studied using two-electrode voltage clamp

methodology. Such procedures enable the detailed study of receptors of known protein composition with a precision and reproducibility difficult to obtain in tissue slices, isolated organs, or whole animals. The results often resemble those obtained from HPLC data more than those usually obtained from living cells! This enables very accurate direct measurements of the functional activity of a range of structurally related ligands on the range of molecularly defined recombinant receptors.

There is a rich diversity of GABA_A receptor proteins represented by five subfamilies that have approximately 30% sequence identity across these subfamilies (Fig. 2). Within these subfamilies there is approximately 70% sequence identity. If we limit all possible combinations of these native GABA_A proteins to those with two α -, two β - and one other subunit (either γ , δ , or ϵ), then there could be more than 2,000 different subtypes of GABA_A receptors. However, the number of major subtypes has been estimated to be less than 10 on the basis of antibody studies [McKernan and Whiting, 1996].

Three GABA_C receptor proteins have been cloned. These show a 30% sequence identity with the GABA_A receptor proteins but do not coexpress with any of the GABA_A receptor proteins, preferring to self-associate into homomeric functional GABA_C receptors that act like those found in the retina. The physiology and pharmacology of homomeric GABA_C receptors are quite distinct from the heteromeric GABA_A receptors [Johnston, 1996b].

The GABA_A and GABA_C receptor families are considered part of a superfamily of fast-acting transmitter-gated ion channel receptors that include nicotinic acetylcholine receptors and strychnine-sensitive glycine receptors that may have evolved from a common ancestor [Ortells and Lunt, 1995; Smith and Olsen, 1995]. Members of this superfamily show a 20% sequence iden-

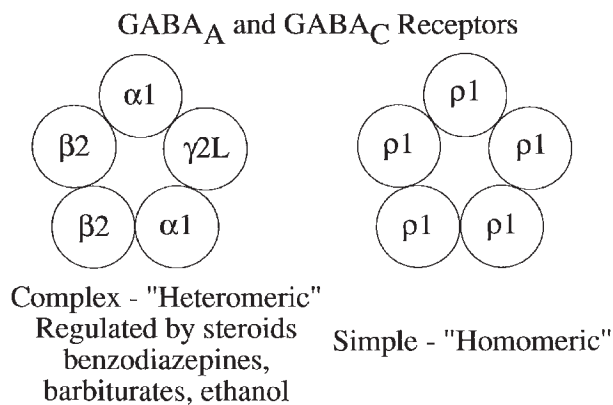


Fig. 1. Diagrams of the pentameric structures of the heteromeric GABA_A and the homomeric GABA_C receptors as seen from outside the cell membrane with the central pores of the pentamers constituting GABA-activated chloride channels.

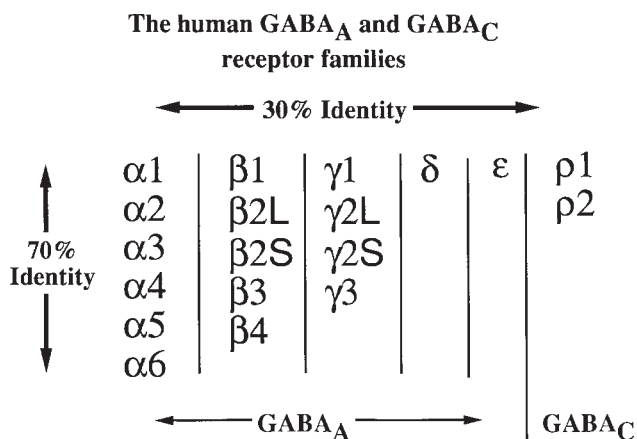


Fig. 2. Table of the protein subunits making up the human GABA_A and GABA_C receptor families and subfamilies. The letters L and S refer to long and short splices variants, respectively.

tity with each other. Each protein subunit appears to have four membrane-spanning regions and these regions represent the highest sequence identity with other proteins in the superfamily.

GABA_B receptors are G-protein receptors negatively coupled to adenylate cyclase made up of a single protein with seven membrane-spanning regions [Kaupmann et al., 1997]. Two GABA_B receptors have been cloned and they show no sequence identity with any of the GABA_A or GABA_C proteins. They show sequence similarity to metabotropic glutamate receptors. The pharmacology of GABA_B receptors is quite distinct from that of GABA_A and GABA_C receptors [Kerr and Ong, 1995].

The predominant GABA_A receptor in mammalian brain is considered to be made up of two α 1, two β 2, and one γ 2L subunit, as shown in Figure 1. This combination of protein subunits may be found in up to 40% of GABA_A receptor complexes in the brain [McKernan and Whiting, 1996]. This combination expressed in *Xenopus* oocytes affords GABA_A receptors in which GABA responses can be modulated by barbiturates, benzodiazepines, and steroids. Change the γ 2L subunit and the modulation by benzodiazepines is lost. The other subunits also influence modulation by benzodiazepines. Thus, the exact mixes of GABA_A subunit combinations are important when considering structure–activity relationships for the modulation of GABA_A receptors by benzodiazepines. Activity datasets for benzodiazepine activity as determined by more conventional pharmacological procedures on mixed populations of GABA_A receptors now need to be treated with some circumspection when used, for example, in QSAR studies (see, e.g., Maddalena and Johnston, 1995). A comprehensive QSAR study on the binding of a range of benzodiazepines to five different recombinant GABA_A receptors has been published recently [Huang et al., 1998].

Many investigators are now studying the effects of different ligands on recombinant GABA_A receptors made up of different combinations of protein subunits (see, e.g., Ebert et al., 1997). In addition to studies on the native protein subunits, studies are being carried out on genetically engineered protein subunits via site-directed mutagenesis (see, e.g., Amin et al., 1997).

GABA_C RECEPTOR SUBTYPES

The recently characterized GABA_C receptors are an important new target in our laboratory for drug development. Two protein subunits have been cloned for GABA_C receptors thus far from human cDNA libraries, named ρ 1 and ρ 2 (consisting of 473 and 465 amino acid residues, respectively). The ρ 1 subunit was first cloned from human retina, where it is predominantly found, while ρ 2 is found more evenly distributed in the CNS. We are interested in developing new chemical entities that interact selectively

with ρ 2 receptors. We have taken advantage of our extensive collection of GABA analogs built up over many years, together with our recently developed ability to study cloned GABA_C receptors expressed in oocytes, to study complementary structure–activity relationships on the ρ 1 and ρ 2 GABA_C receptor subtypes.

Our initial studies on ρ 1 receptors showed that compounds with substituents at the C2 position of GABA and its unsaturated analog trans-4-aminocrotonic acid were well tolerated, but substituents elsewhere resulted in dramatic loss of activity [Chebib et al., 1997]. Molecular modeling suggested that the agonist/competitive antagonist binding site of the ρ 1 GABA_C receptor may be smaller than that of the GABA_A and GABA_B receptors, accommodating only compounds that can attain relatively flat conformations.

Differences were found between ρ 1 and ρ 2 receptor pharmacology, with antagonists being generally more potent against ρ 1 than ρ 2 receptors and the reverse being true for agonists [Chebib et al., 1998]. The most interesting compound in this study was trans-4-amino-2-methylbut-2-enoic acid, a ρ 1 antagonist (K_B 45 μ M) but a partial agonist at ρ 2 receptors, with an intrinsic activity 33% of that of the maximum response of GABA. This compound provides a lead to the development of new chemical entities that can clearly distinguish between the ρ 1 and ρ 2 GABA_C receptor subtypes.

Human GABA_C ρ 1 and ρ 2 receptor subunits show an overall 74% sequence identity, with a 20% sequence divergence in the N-terminal domain [Cutting et al., 1992]. Pharmacological differences between receptor subtypes can occur with only a single key amino acid residue difference. In rat GABA_C receptors, there is a methionine in the putative second transmembrane domain present in the ρ 2 but not in the ρ 1 subunit, which is critical for resistance to antagonism by picrotoxin [Zhang et al., 1997].

The stage is now ready for extensive studies on structure–activity relationships in GABA_C ρ 1 and ρ 2 receptor subunits that have been further modified by site-directed mutagenesis. A further advance will be the use of chimeric proteins constructed from the native human ρ 1 and ρ 2 receptors, together with the ρ receptors cloned from other species: three from rat, two from chick, two from mouse, and five from white perch retina thus far. This chimeric methodology is illustrated by our studies on glutamate transporters.

GLUTAMATE TRANSPORTERS

A number of different glutamate transporters have been identified, initially by pharmacological studies and subsequently by molecular biological techniques [Vandenberg, 1998]. Five glutamate transporter subtypes have been cloned; the human clones have been termed

EAAT1 to EAAT5 (*excitatory amino acid transporters* 1–5). There is between 40 and 65% sequence identity between the five cloned transporters and they are thus likely to form similar structures in membranes.

Of the five EAATs cloned thus far, only EAAT2 is sensitive to inhibition by kainate (K_i 17 μM), a conformationally restricted glutamate analog. This is much more sensitive than is observed in rat brain slices, where kainate was shown to be a competitive inhibitor (K_i 250 μM) of glutamate uptake [Johnson et al., 1979]. Thus, pharmacological studies suggest that there may be a “missing” kainate-sensitive transporter yet to be cloned. This transporter could be from a different family of transporters with little sequence identity with the EAAT family of transporters.

There is uncertainty regarding the membrane-spanning topology of the EAAT family of transporters based on hydrophobicity analysis and other studies. Two fundamentally different structures have been proposed, one containing ten transmembrane α -helical domains [Slotboom et al., 1996] and one containing six α -helical domains and four transmembrane β -sheets [Wahle and Stoffel, 1996]. Even though different transporter subtypes can exist in the same cell, chemical crosslinking studies suggest that they form only homomeric transporters [Haugeto et al., 1996]. Monomers, dimers, and trimers of the transporter proteins have been detected in reconstituted liposomes but the stoichiometry of the functional transporters is not yet clear.

The EAAT1, EAAT2, and EAAT3 glutamate transporter subtypes are found throughout the brain. EAAT1 is the most abundant of the cloned transporters in the cerebellum and is expressed in both glial cells and neurones. EAAT2 is the most abundant in other regions of the brain and is found exclusively in glial cells. EAAT3 is neuronal and highly expressed in the cerebral cortex, hippocampus, and caudate-putamen. EAAT4 is expressed in the Purkinje cell layer of the cerebellum, while EAAT5 is expressed mainly in the retina.

EAAT1 AND EAAT2 LIGANDS

EAAT1 and EAAT2 show 65% sequence identity. If conservative substituents are allowed, the degree of relatedness is increased to 80% [Arriza et al., 1997]. These two proteins are thus likely to form very similar structures, but they show quite distinct physiological and pharmacological differences when expressed in *Xenopus* oocytes.

Both EAAT1 and EAAT2 are able to transport L-glutamate, L-aspartate, and D-aspartate as high affinity substrates. Substrates for EAAT1 and EAAT2 are, in general, small flexible molecules, which may be a requirement for passage through the pore of the transporter. Transport of L-aspartate and D-aspartate by EAAT1 is

accompanied by a large chloride conductance that is thermodynamically uncoupled to amino acid transport. By comparison, the chloride conductance activated by L-glutamate transport by EAAT1 is significantly smaller, while transport of L-glutamate, L-aspartate, and D-aspartate by EAAT2 allow very little chloride ion flux [Wadiche et al., 1995]. L-serine-O-sulfate is a high-affinity substrate for EAAT1, but is approximately 8-fold less potent as a substrate for EAAT2. In addition, L-serine-O-sulfate transport by EAAT2 shows a steep voltage dependence, suggesting that the pore of EAAT2 is different from that of EAAT1 [Vandenberg et al., 1998].

Kainate, dihydrokainate, and threo-3-methylglutamate are high-affinity competitive inhibitors of transport by EAAT2, with little effect on transport by EAAT1. (2S,4R)-4-methylglutamate and 4-methylene-glutamate are substrates for EAAT1 but inhibitors of EAAT2, while L-threo-4-hydroxyglutamate is a substrate for both EAAT1 and EAAT2 [Vandenberg et al., 1997]. Thus, both the chemical nature and the orientation of substituents at the 4-position of glutamate are critical in determining the nature of interaction with these two glutamate transporters. It seems that EAAT1 and EAAT2 recognize methyl- and hydroxyl-derivatives of glutamate in subtly different ways.

CHIMERIC TRANSPORTERS

The physiological and pharmacological differences between EAAT1 and EAAT2 raise the question as to which part of the transporter proteins are responsible for these differences. The most highly conserved domains are most likely to be important for the functional properties of the transporters. Thus, it may be predicted that amino acid changes within these conserved domains play important roles in determining some of the physiological and pharmacological differences. One way to identify the possible amino acid residues determining these differences is to construct a series of chimeric transporters using EAAT1 (542 amino acid residues) and EAAT2 (574 amino acid residues) as templates. (The term “chimeras” comes from a fire-breathing monster in Greek mythology having a lion’s head, a goat’s body, and a serpent’s tail.)

Two procedures were used to construct the various chimeras. In one procedure, unique restriction sites were engineered into the cDNAs of EAAT1 and EAAT2 using a site-directed mutagenesis kit, and then the cDNA fragments of one of the transporter were cut out of the plasmid and ligated into the corresponding sites of the other transporter cDNA [Mitrovic et al., 1998]. In the other procedure, recombinant chimeras were generated by a restriction site-independent method using an *in vivo* recombination methodology as described by Buck and Amara [1994].

The five chimeras illustrated in Fig. 3 were useful for investigating the importance of transmembrane domains 7–10 in determining functional differences between EAAT1 and EAAT2. They represent chimeric transformations between EAAT1 and EAAT2 in four regions: region 1, starting at the amino terminus and including the first six transmembrane domains and part of transmembrane domain 7; region 2, continuing through transmembrane domain 7 to the end of transmembrane domain 8; region 3, including transmembrane domains 9 and 10; and region 4, consisting of the intracellular carboxyl-terminal region (Fig. 3).

Our studies on these chimeras defined a region including transmembrane domains 9 and 10 to be of primary importance in determining the functional differences between EAAT and EAAT2. This region consists of 57 amino acids, of which 17 are not conserved between EAAT1 and EAAT2. Three of the 17 amino acids have been allocated to extracellular domains, ten are located in the two transmembrane domains, and four are positioned intracellularly according to the ten transmembrane domain model of Slotboom et al. [1996]. It can be predicted that one or more of the nonconserved 17 amino acids in transmembrane domain 9 and transmembrane domain 10 contribute to the differences between native EAAT and EAAT2 in L-serine-O-sulfate substrate selectivity, chloride permeability, and sensitivity to inhibitors such as kainate, and that transmembrane domains 9 and 10 form part of the pore region of the transporters or that they have an interactive role modulating the function of the pore re-

gion [Mitrovic et al., 1998]. The nonconserved 17 amino acids in transmembrane domain 9 and transmembrane domain 10 of EAAT1 and EAAT2 now become targets for more detailed site-directed mutagenesis studies.

CONCLUSION

This overview highlights the importance of interactions between medicinal chemists, molecular biologists, and pharmacologists in developing techniques with which to study molecular aspects of drugs and their protein targets. While variation of the structure of drug leads is well developed, variation of the structure of the protein targets is in its infancy. For membrane bound receptors and transporters, we are mainly at the level of manipulating the primary amino acid sequence. Clearly, we need more information relating to the three-dimensional structures of these drug targets: molecular modeling is providing some information on this aspect (see, e.g., Gready et al., 1997; Ortells et al., 1997).

Subtypes of receptors and transporters are important drug targets. There is a remarkable diversity of such targets, particularly those made up of different mixtures of protein subunits in heteromeric receptors. We need a better understanding of the relative importance of individual subtypes both under normal conditions and in disease states. Mutations of receptors and transporters in disease states appear to be relatively uncommon, but inheritable mutations in the superfamily of transmitter-gated ion channels that includes GABA_A, glycine, and nicotinic acetylcholine receptors have been described [Vafa and Schofield, 1998]. Complementary structure–activity relationships between drugs and their protein targets will provide a greater understanding of drug action at the molecular level and facilitate development of drugs that interact selectively with particular native and mutant protein receptor/transporter subtypes.

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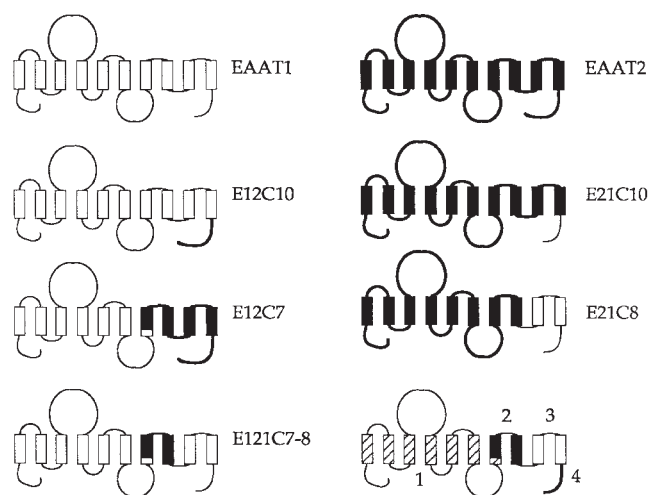


Fig. 3. Diagrams of the structures of the native EAAT1 and EAAT2 transporters, and some chimeric transporters constructed from them. The heavy lines and filled rectangles depict areas in the chimeras derived from EAAT2. The four regions probed in these studies are indicated in the bottom right diagram. Region 3, which contains transmembrane domains 9 and 10, is of primary importance in determining functional differences between the transport subtypes.

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