Functional and Pharmacological Aspects of GABA Transporters

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18 Functional and Pharmacological Aspects of GABA Transporters

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1 GABA: An Introduction

AU1

Almost 60 years ago, three research groups independently discovered that GABA is present in the brain (Awapara et al., 1950; Roberts and Frankel, 1950; Udenfriend, 1950). Subsequently, it has been established as the major inhibitory neurotransmitter in the mammalian central nervous system (CNS) (Elliott and van Gelder, 1958; Krnjevic and Schwartz, 1967; Roberts, 1971; Curtis and Johnston, 1974). GABA is estimated to be present in 60–75% of the synapses in the CNS (Durkin et al., 1995), hence it plays a significant role in the maintenance of the normal function in the CNS, which is established in concerted action with the major excitatory neurotransmitter, glutamate.

Dysfunction in the CNS neurotransmission, resulting from an imbalance between the inhibitory and excitatory currents, manifests itself in numerous pathological diseases among others, epilepsy. Disinhibition is considered to be the precipitating factor in epilepsy, leading to hyperexcitable neurons which discharge in a highly synchronized manner propagating into a full seizure (Lloyd and Morselli, 1987; De Deyn et al., 1990; Dalby and Mody, 2001). Although this imbalance theory is a gross oversimplification, several experimental results strengthen it; reduced GABA levels in human tissue surgically removed from patients with intractable epilepsy have been found and moreover, the chemoconvulsants pentylentetrazol (PTZ) and methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) reduce the GABA response in a dose-dependent manner (De Deyn et al., 1990). Benzodiazepines and barbiturates enhance the GABA-mediated inhibitory neurotransmission, and tiagabine and vigabatrin increase the synaptic GABA concentration, all of which display an anticonvulsant effect.

2 GABAergic Neurotransmission

GABA is synthesized in neurons by a pathway, often referred to as the "GABA shunt." In short, α-ketoglutarate is funneled out of the tricarboxylic acid cycle and transaminated by GABA transaminase (GABA-T) to L-glutamate which is converted to GABA by glutamate decarboxylase (GAD) (Roberts and Kuriyama, 1968; Balazs et al., 1970). GAD exists in two forms with molecular weights of 67 and 65 kDa referred to as GAD_{67} and GAD_{65}, respectively. GAD_{67} and GAD_{65} differ in subcellular localization; the former being distributed throughout the neuron but primarily in the soma and dendrites, the latter is predominantly located at the nerve endings near the synaptic vesicles. GAD is dependent on its cofactor pyridoxal 5′-phosphate (PLP) for activity. Nearly all GAD_{67} is in the activated holoenzyme form, whereas GAD_{65} is only partly saturated with PLP (Kaufman et al., 1991). Due to the different localizations of GAD_{67} and GAD_{65}, and the fact that GAD_{65} is mainly in the apoenzyme form (inactive, without bound PLP) and readily inducible by neuronal activity, it has been proposed that GAD_{65} is responsible for maintaining adequate amounts of GABA in the nerve terminal during heightened neuronal activity (Erlander and Tobin, 1991; Kaufman et al., 1991).

GABA is predominantly packaged into vesicles by a vesicular GABA transporter (VGAT), which uses the proton gradient to drive the uphill transport, and released into the synaptic cleft in a Ca^{2+}-dependent manner (Nicholls, 1989). However, GABA can also be released into the cleft via reversal of the GABA transporters present on the presynaptic neuron (During et al., 1995).

Upon release to the synaptic cleft, GABA interacts with $GABA_A$, $GABA_C$, and $GABA_B$ receptors; the first two being ionotropic and primarily located postsynaptically and the latter being metabotropic and localized both pre- and postsynaptically (Feldman et al., 1997; Watanabe et al., 2002). The termination of the GABAergic neurotransmission is facilitated via high-affinity transport proteins located in both presynaptic neurons and surrounding glia cells (Iversen and Neal, 1968; Iversen and Kelly, 1975). Upon entry into the presynaptic neuron and glia cells, GABA is degraded by the catabolic enzymes GABA-T and succinate semialdehyde dehydrogenase (SSADH) to succinate, which reenters the TCA cycle (Waagepetersen et al., 2003) and completes the "GABA shunt." It has been estimated that roughly 8–10% of the flux through the TCA cycle in GABAergic neurons is accounted for by the GABA shunt (Balazs et al., 1970).

3 GABA Transporters

With the establishment of GABA as an inhibitory neurotransmitter and the elucidation of its metabolism as described above, a more detailed elaboration on the GABA transporters as pharmacological targets is warranted. Elliott and van Gelder (1958) were among the first to determine that GABA in the incubation medium could accumulate into slices of cerebral cortices. However, it was not until 10 years that the existence of high-affinity transport proteins for GABA was discovered in both neurons and astrocytes (Iversen and Neal, 1968). Later, it was shown that ^{3}H-GABA in rat cerebellum was predominantly accumulated not only into stellate cells or other interneurons but also into astrocytes (Hösli and Hösli, 1976, 1978). Diaminobutyric acid (DABA) and 3-hydroxy-5-aminovaleric acid have been shown to be selective inhibitors of neuronal GABA transport, whereas β-alanine and β-proline are selective inhibitors of glial GABA transport (Iversen and Kelly, 1975; Schousboe et al., 1979). The neuronal GABA uptake inhibitors DABA and *cis*-3-aminocyclohexane carboxylic acid (ACHC) have furthermore been shown to be proconvulsive (Krogsgaard-Larsen, 1981). Taking this into account in combination with the notion that approximately 20% of synaptically released GABA is taken up into astrocytes, where it is subsequently degraded to succinate and lost from the GABA pool, the hypothesis has been proposed that selective inhibition of astrocytic GABA transport would increase the amount of GABA recycled into neurons, enhancing the GABAergic neurotransmission, thereby preventing the generation of seizures (Schousboe et al., 1983; Schousboe, 1990, 2000).

3.1 Cloning of High-Affinity GABA Transporters

In an effort to clone the neuronal and glial GABA transporter further intricacies have been revealed. Radian et al. (1986) were the first to isolate an 80-kDa glycoprotein transporter from rat with a Na^+ and Cl^- dependence for transport and an apparent K_m for GABA of 3 μM. Subsequently, this transporter was cloned and designated GAT-1. It revealed a 67-kDa transporter protein consisting of 599 amino acids with an absolute dependence of Na^+ and Cl^- for transport and a K_m of 7 μM for GABA. Pharmacological characterization of previously established inhibitors of either neuronal or glial GABA transport revealed a pharmacology related to the neuronal subtype (Guastella et al., 1990). The human GAT-1 has also been cloned and consists of 599 amino acids as well (Nelson et al., 1990).

A transport protein capable of transporting both GABA and the osmolyte betaine with an apparent K_m of 93 and 398 μM, respectively, was isolated from rat kidney and named the betaine-GABA transporter 1 (BGT-1). It encodes a 614 amino acid protein also with a dependence on Na^+ and Cl^- for transport. The neuronal GAT inhibitors nipecotic acid and DABA display low affinities for the BGT-1 (Yamauchi et al., 1992). The human BGT-1 has also been cloned and consists of 614 amino acids and is highly dependent on Na^+ and Cl^- for transport. In contrary to rBGT-1, the human clone displays a 25-fold higher affinity for GABA than for betaine. Localization studies in eight brain regions revealed the presence of hBGT-1, but it did not match GABAergic pathways closely; hence, it was concluded that it may not terminate GABA
AU2 neurotransmission but rather sequester GABA diffused away from the synapsis (Borden et al., 1995; Rasola et al., 1995).

Two more rat GAT clones designated rGAT-2 and rGAT-3 with a K_m of 8 and 12 μM for GABA, respectively, and an amino acid sequence of 602 and 627, respectively, have also been cloned. Both GAT-2 and GAT-3 display a pharmacological profile different from that of the previously reported clones. β-Alanine has a high affinity toward both clones, whereas nipecotic acid and DABA have low affinities, suggesting a higher resemblance to the glial GABA transporter (Borden et al., 1992). Later, the human GAT-3 clone of 632 amino acids was discovered (Borden et al., 1994).

Four mouse GABA transporters displaying a Na^+ and Cl^- dependence for transport have been cloned and characterized pharmacologically. GAT1, GAT2, GAT3, and GAT4 (without hyphen) are composed of 598, 614, 602, and 627 amino acids, respectively. Interestingly, nipecotic acid, DABA, and guvacine are more

potent inhibitors of GAT1, GAT3, and GAT4 than of GAT2. Betaine only inhibits GAT2, whereas β-alanine preferentially inhibits GAT3 and GAT4 (Liu et al., 1993).

The nomenclature of the GATs among species seems rather confusing and therefore some clarification may be warranted. The nomenclature between rat and human clones is the same, but when comparing to the mouse clones problems appear. Consequently, rat GAT-1, BGT-1, GAT-2, and GAT-3 correspond to mouse GAT1, GAT2, GAT3, and GAT4, respectively (see *Table 18-1* for references). In the following, the nomenclature according to the mouse clones will be used in general terms when referring to homologous GABA transporters between species. To characterize these clones, the respective authors listed above

Table 18-1
GABA transporter nomenclature across species

Species	Nomenclature			
Rat	GAT-1[a]	BGT-1[b]	GAT-2[c]	GAT-3[c]
Human	GAT-1[d]	BGT-1[e]	NC	GAT-3[f]
Mouse	GAT1[g]	GAT2[g]	GAT3[g]	GAT4[g]

[a]Guastella et al. (1990)
[b]Yamauchi et al. (1992)
[c]Borden et al. (1992)
[d]Nelson et al. (1990)
[e]Borden et al. (1995)
[f]Borden et al. (1994)
[g]Liu et al. (1992), Liu et al. (1993)
NC not cloned

expressed the clones in relevant expression systems and tested their pharmacology. However, due to the various compounds tested and the unavailability of all the respective IC_{50} values between their works, the pharmacology of GAT1–4 and neuronal and glial GAT is summarized in *Table 18-2*. *Table 18-2* displays the IC_{50} values for the two proposed neuronal inhibitors, namely DABA and ACHC, and the glial inhibitor β-alanine as well as nipecotic acid, all tested on the mouse clones expressed in HEK-293 cells and on primary cultures of cortical neurons and astrocytes from mice.

In summary, four GABA transporters are expressed on the plasma membrane of neurons and astrocytes. GAT1 represents a pharmacology closely related to neuronal GAT. GAT3 and GAT4, which displayed the highest affinity for β-alanine and lower affinity for nipecotic acid, DABA, and ACHC, were thought to represent the glial GAT. GAT2 is quite distinct from the other GATs, although being a high-affinity GABA transporter; GAT2 is the only GABA transporter capable of transporting the osmolyte betaine. The K_m for betaine is ∼398 μM, which is fourfold higher than the K_m for GABA (Yamauchi et al., 1992). However, the above description of the GABA transporter substrate specificity has revealed a much less straightforward relationship between neuronal and glial GATs and the cloned GAT1–4 than would have been expected or hoped. To further appreciate this dilemma, the subcellular localization will briefly be outlined in the following section.

3.2 Subcellular Localization of GABA Transporters

GAT1 is found to be expressed on the apical surface in polarized MDCK cells (Pietrini et al., 1994), which is in agreement with GAT1 being restricted to axonal segments in cultured neurons. Moreover, GAT1 expression closely reflects GABAergic pathways (Radian et al., 1990; Pietrini et al., 1994; Borden, 1996;

Table 18-2
Inhibitory activities of various GABA analogs on cortical neurons and astrocytes, and cloned mouse GAT1–4

	GABA uptake inhibition IC_{50} or $^{*}K_{m/i}$ (μM)					
Compound	Neurons	Astrocytes	GAT1	GAT2	GAT3	GAT4
GABA	8[a]	32[a]	17[a]	51[a]	15[a]	17[a]
Nipecotic acid	12	16	24	>1000	113	159
Guvacine	32	29	39	>1000	228	378
DABA	1000	>5000	128	528[c]	300	710
ACHC	200	700	132	1070[c]	>1000	>10000
β-Alanine	1666[b]	843[b]	2920	1100[c]	66	110
THPO	501[b]	262[b]	1300	3000	800	5000
Exo-THPO	780	250	1000	3000	>3000	>3000
N-Methyl-*exo*-THPO	405	48	450	>3000	>3000	>3000
N-Ethyl-*exo*-THPO	390	301	320	>1000	>1000	>1000
N-2-Hydroxyethyl-*exo*-THPO	300	200	>500	>500	>500	>500
N-4-Phenylbutyl-*exo*-THPO	100	15	7	>500	>1000	>1000
N-Acetyloxyethyl-*exo*-THPO	200	18	550	>1000	>1000	>1000
(*R*/*S*)-EF1502	2	2	7	26	>300	>300
(*R*)-EF1502	1.5	0.65	4	22	>150	>150
(*S*)-EF1502	>100	>100	120	34	>150	>150
N-DPB-THPO	38[b]	26	30	200	>300	>1000
N-DPB-Nipecotic acid	1.3[b]	2.0[b]	0.64	7210[c]	550	4390
N-DPB-guvacine	4.9[b]	4.2[b]	–	–	–	–
N-DPB-*exo*-THPO	1.4	0.6	6	100	>100	>100
N-DPB-*N*-Methyl-*exo*-THPO	5	2	2	200	>100	>100
NNC 05-2090	–	–	19	1.4	41	15
SNAP-5114	–	–	>30	22	20	6.6
NNC-711	1.24	0.64	–	–	–	–
Tiagabine	0.45	0.18	0.11	>100	>100	800

*Data summarized from Bolvig et al. (1999), Borden (1996), Clausen et al. (2005), Falch et al. (1999), Larsson et al. (1981, 1983, 1986, 1988), Sarup et al. (2003a), Schousboe (1979), Suzdak et al. (1992), Thomsen et al. (1997), White et al. (2002)

[a] K_m

[b] K_i

[c] Human BGT-1

Conti et al., 1998). Furthermore, the cell body of neurons is devoid of GAT1 (Radian et al., 1990). The distal processes of glial cells are also labeled with GAT1, and they are in close proximity of axons forming symmetric synapses; however, the staining intensity of glial GAT1 is much lower than that seen in neurons (Conti et al., 1998).

GAT2 shows a basolateral targeting in polarized MDCK cells (Pietrini et al., 1994; Ahn et al., 1996) consistent with dendritic and cell body labeling in hippocampal neurons (Zhu and Ong, 2004a). Contrary to GAT1, GAT2 was found not to be located close to GABAergic synapses, rather the dendrites make asymmetric contact with glutamatergic neurons. Moreover, GAT2 was observed to be located in the extrasynaptic region (Borden et al., 1995; Zhu and Ong, 2004a). GAT2 is also found on glia cells and in primary cultures of astrocytes (Zhu and Ong, 2004b; Olsen et al., 2005).

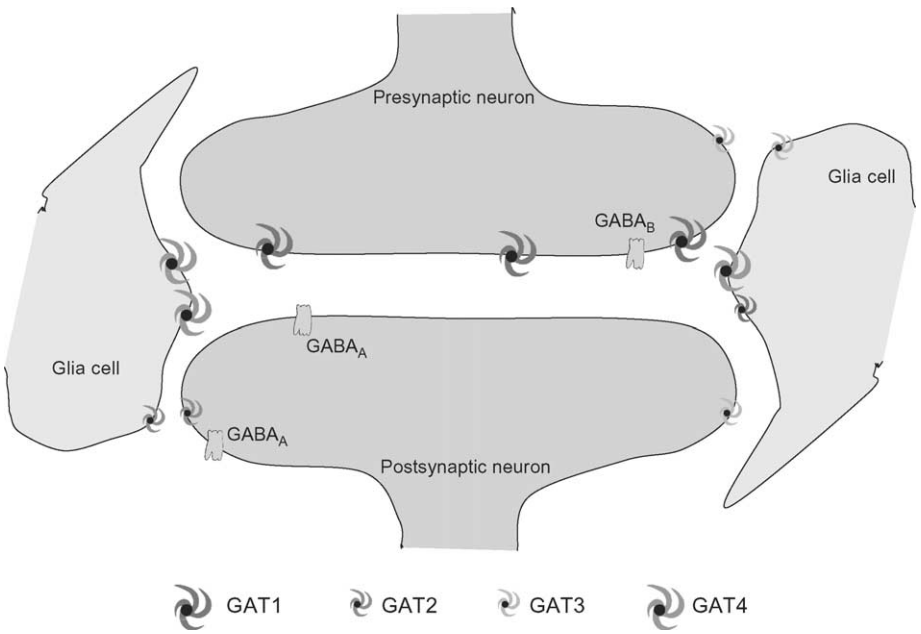
GAT3 is primarily expressed on the basolateral surface of polarized MDCK cells (Ahn et al., 1996), suggesting a dendritic localization on neurons. Furthermore, GAT3 is found on distal astrocytic processes. It is concluded that GAT3 is primarily located in the extrasynaptic region (Conti et al., 1999, 2004). GAT4 is

localized to the apical membrane of polarized MDCK cells (Ahn et al., 1996). GAT4 is localized to both neurons and astrocytes; however, it is primarily localized to the latter cell type (Durkin et al., 1995; Minelli et al., 1996).

For a more detailed overview of the GAT localization in different brain regions, the reader is referred to Durkin et al. (1995) which contains a thorough presentation of GAT1, GAT3, and GAT4. For GAT2, the reader is referred to the above-mentioned works of Zhu and Ong (2004a, b). In summary, GAT1 and GAT4 are highly abundant and oppositely distributed within the CNS between neurons and astrocytes, respectively, primarily within the synapses. GAT2 and GAT3 are far less abundant and primarily expressed in the extrasynaptic region; these localizations are summarized in *Figure 18-1*.

Figure 18-1

Shown is a summary of the subcellular localization of GAT1–4. For convenience, they are represented in the same synapsis, although it is a great simplification. (Madsen et al., 2006—with kind permission of Springer Science and Business Media)



3.3 Structure

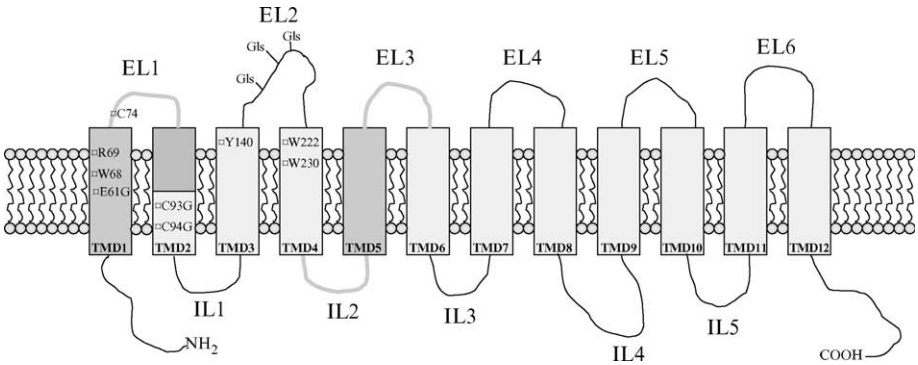
The GABA transporters belong to the SLC6 superfamily of Na^+-dependent transporters that can be divided into four groups: (1) Transporters of the biogenic amines noradrenalin, dopamine, and serotonin; (2) various GABA transporters as well as transporters of taurine and creatine; (3) transporters of proline and glycine; and (4) "orphan" transporters.

Common to all the transporters is the similar secondary structure composed of 12 putative hydrophobic transmembrane domains (TMD) which are connected by intracellular loops (IL) and extracellular loops (EL) (Guastella et al., 1990; Kanner, 1994). Within the SLC6 family, highly conserved regions exist which

include TMD1, EL1, part of TMD2, IL2, TMD5, and EL3 (Kanner, 1994); these regions are recognized as gray regions in *Figure 18-2*.

Figure 18-2

Summarized drawing of the 12-TMD structure of GAT1, showing in gray the conserved domains/loops in the SLC6 transporter family, and a presentation of the discussed amino acids. Furthermore, the three glycosylation sites (Gls) are shown on EL2. Both the N and C terminus are positioned intracellularly



The GATs are as previously described dependent on sodium and chloride ions for transport and the stoichiometry for transport is 2–3 sodium ions:1 chloride ion:1 GABA molecule (Keynan and Kanner, 1988). GATs are capable of generating a gradient in the order of 10^5 between the intra- and extracellular GABA concentration (Beleboni et al., 2004). About 30–65% amino acid sequence homology exists between different SLC6 family members. For a more thorough review of the amino acid sequence relationship within the SLC6 transporter superfamily please see Miller et al. (2002).

Several mutation studies have been made in an effort to elucidate specific amino acids involved in the binding of GABA and other substrates and also the regions either TMDs or IL or EL that are involved in lining the GABA binding pocket. Furthermore, several studies address the substrate specificity of GAT1–4 in-between one another. These amino acids/domains are also shown in *Figure 18-2*.

Arginine R69 that bears a positive charge is essential for transport, since its substitution with other amino acids including charged ones does not recover transport activity. It has been suggested that R69 plays a role in binding of chloride ions (Pantanowitz et al., 1993). Tryptophan W68, W222, and W230 when substituted with either serine or leucine resulted in a 90% reduction in transport activity. It appears that W68 and W222 are required for intrinsic activity. Moreover, W230 has been reported to be involved in plasma membrane targeting (Kleinberger-Doron and Kanner, 1994). Tyrosine Y140 replacement is not tolerated even with the aromatic amino acids phenylalanine or tryptophan. It is speculated that this residue interacts with the amino group of the amino acids and biogenic amines of the SLC6 gene family (Bismuth et al., 1997). Cysteine C74 located in EL1 is also suggested to be involved in the pore formation or GABA translocation (Yu et al., 1998).

The three proposed glycosylation sites in EL2 have been confirmed, and it was found that 40% of the transport activity remained after deletion of these sites. Furthermore, via the introduction of two glycosylation sites in EL3, the transport activity was abolished; hence the glycosylation sites in EL2 are highly specific and important for transport activity. However, using *N*-glycosylation a discrepancy in the theoretical transmembrane model occurred since IL1 could be glycosylated in vivo, suggesting an extracellular position of IL1. Moreover, evidence suggests that EL1 actually resides intracellularly (Bennett and Kanner, 1997). Yu et al. (1998) found that the EL1 is located extracellularly quite contrary to Bennett and Kanner

(1997). However, they also found that IL1 should be located extracellularly. To accommodate this new topology, the TMD2 is proposed not to span the entire membrane rather to make a reentrant loop which accommodates the extracellular facing of IL1. Furthermore, a new TMD3′ is added which is made up of residues toward the N termini of EL2, thereby TMD3 and TMD3′ are connected by the new IL1 (Yu et al., 1998). Clark (1997) has proposed a further adaptation of the 12TMD structure of the GABA transporter. Through protease protection studies, they found that EL2 and EL4 are accessible to cytoplasmatic protease activity. This can be explained by EL2 + 4 making a pore loop structure into the membrane and out again. These loops might be involved in the substrate-binding pocket (Clark, 1997). Evidence regarding EL4 involvement in binding and translocation of sodium and GABA has further been established by Zomot and Kanner (2003). Furthermore, TMD1 and TMD3 are found to be in close proximity within the transporter and are involved in the formation of the binding pocket and participate in the translocation pathway, especially the extracellular half of TMD1 that is thought to have a more extended structure than a normal α-helix which would facilitate these actions (Zhou et al., 2004; Zomot et al., 2005). In this context, the recently published crystal structure of a bacterial leucine transporter (Yamashita et al., 2005) homologous to the GABA transporter has provided important structural information (see below), but detailed knowledge about the topology of the bona fide GABA transporters will have to await the availability of crystal structures before precise conclusions can be made.

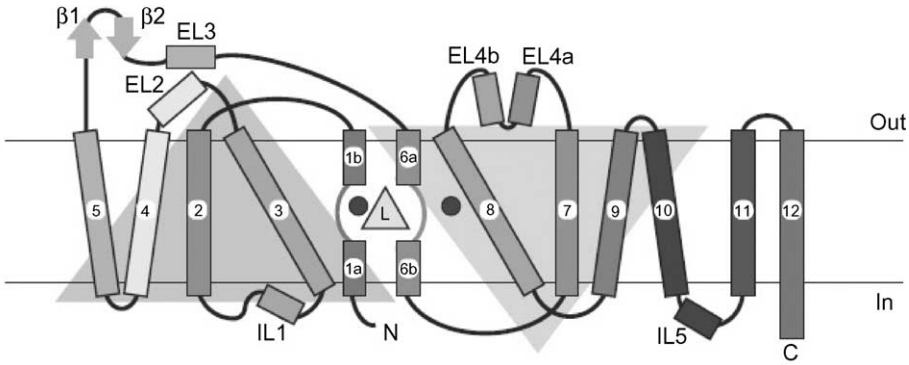
Mutagenic studies have revealed a functional role for TMD1 + 2 in shaping the substrate-binding pocket of GAT4. The E61G/C93G/C94G mutant shows a 30-fold decrease in K_i for taurine toward the wild-type transporter. The fact that it competitively inhibits GABA transport and that the sizes of the side chains in E61 and C94 are inversely related to the potency of taurine suggests an indirect effect on the GABA binding pocket in GAT4 (Melamed and Kanner, 2004). In another study conducted by Tamura et al. (1995), site-directed mutagenesis was utilized to introduce the EL3 + 5, EL4, and EL6 of GAT3, GAT4, and GAT2, respectively, into GAT1 and the EL5 domain of GAT1 into that of GAT3 to determine the substrate specificity conveyed by these extracellular domains. The introduction of EL3 and EL5 of GAT3 into GAT1 resulted in a significant increase in V_{max} of 2.5 and 4, respectively, but the K_m was not influenced to any significant extent. Furthermore, the EL5 mutant of GAT1 displayed a shift in the β-alanine sensitivity. Moreover, the reverse mutant of GAT3 became less sensitive to β-alanine, suggesting a role of EL5 in the substrate binding of β-alanine. The EL4 GAT1 mutant revealed a fourfold decrease in K_m. The EL6 GAT1 mutant led to a ninefold higher K_m and a twofold increase in V_{max} when compared to the wild-type GAT1. These data suggest that EL4, EL5, and EL6 are involved in the substrate-binding pocket.

The crystal structure of a bacterial homolog of a Na^+/Cl^--dependent transporter (Yamashita et al., 2005), namely the Na^+-dependent leucine transporter from *Aquifex aeolicus* ($LeuT_{Aa}$), reveals interesting structural insight into the SLC6 transporter family. Even though the prokaryotic and eukaryotic only share 20–25% sequence homology in the primary structure within this family, the data is still very relevant. $LeuT_{Aa}$ consists of 12 TMD, and the transporter is shaped like a shallow "shot glass" ~70-Å tall and ~48 Å in diameter. The authors identified a repeat in the first ten TMDs, TMD1–5 and TMD6–10 form a pseudo twofold symmetry along the axis of the membrane, and the two stretches are positioned opposite to one another. A break in the α-helical structure in TM1 and TMD6 from V23-G24 and S256-G260, respectively, exposes the carbonyl oxygen and nitrogen atoms, which are then available for hydrogen bonding and ion coordination in the substrate-binding pocket. Contributing to this effect are TMD3 and TMD8, which display a highly conserved stretch of amino acids surrounding the breaks. These TMDs are thought to represent the core of the transporter. Furthermore, EL2 and EL4, which are juxtaposed across from each other, form the rim of the "shot glass." EL2, TMD9, and TMD12 are also involved in dimerization of the $LeuT_{Aa}$ (Yamashita et al., 2005). The crystal structure of $LeuT_{Aa}$ is shown in ❯ *Figure 18-3*. TMD1, which is obviously a part of the leucine-binding pocket, and EL2 form part of the rim of the transporter along with its involvement in dimerization, and these two domains present interesting features in the bacterial Leu_{Aa}, especially because they are highly conserved in the SLC6 transporter family.

AU3

The fact that EL2 and EL4 were reported by Clark (1997) to be involved in the substrate-binding pocket, and that TMD1 and TMD3 were suggested by Zhou et al. (2004) and Zomot et al. (2005) to be involved in the binding pocket of the transporter, draws direct comparisons to $LeuT_{Aa}$.

Figure 18-3
Crystal structure of $LeuT_{Aa}$. Light gray *rectangle* represents leucine and the *filled circles* represent sodium ions. [Reprinted by permission from Macmillan Publishers Ltd: [Nature] (Yamashita et al., 2005), copyright (2005)]



3.4 Trafficking

Studies on the regulation of GAT1 in regard to trafficking have recently been reviewed by Robinson (2002). In the following section, a brief outline of the trafficking behavior of GAT1 will be presented. Activation of protein kinase C (PKC), which can be induced by agonists on different G protein–coupled receptors, results in a decreased cell surface expression of GAT1 in primary cultures of hippocampal neurons (Beckman et al., 1999). Inhibition of tyrosine kinase results in a reduction of the transport activity of GAT1, which correlates with a reduction in V_{max} but not K_m, hence a reduced surface expression of GAT1. These changes are occurring simultaneously with an average reduction of 54% of GAT1 specific tyrosine phosphorylation. Moreover, inhibition of tyrosine phosphatase increases the surface expression of GAT1 and prevents the decrease caused by tyrosine kinase. These data suggest that a balance between tyrosine kinase and phosphatase regulates GAT1 expression. Furthermore, tyrosine kinase/phosphatase and PKC regulation of GAT1 seems to occur through different mechanisms (Law et al., 2000). The regulation of GAT1 also occurs via a transport-mediated process in which GABA and the two substrates nipecotic acid and ACHC cause an upregulation in GAT1 transport activity. This is visualized by an increase in V_{max} and surface expression of GAT1. The nontransportable and competitive inhibitor of GAT1-mediated transport, SKF89976A (Larsson et al., 1988), shows the exact opposite pattern when compared to GABA. Furthermore, intracellular levels of GABA do not regulate GAT1 expression. This suggests that extracellular GABA halts the internalization of GAT1 (Bernstein and Quick, 1999).

GAT1 expressed on the surface is internalized in a clathrin-mediated endocytosis as are synaptic vesicles. Furthermore, GAT1 recycling is regulated in a Ca^{2+}-dependent manner. GAT1-containing vesicles are normally distributed with a diameter of 47 ± 13 nm similar to synaptophysin-containing vesicles. However, they lack synaptophysin and the VGAT, suggesting that they comprise a distinct class of vesicles (Deken et al., 2003). The same research group found that about one third of GAT1 in the cell constitutes the acutely recycling pool, of which one third (about 300 transporters) are expressed on the cell surface in the basal state. Kinetic analysis has revealed the endocytosis and exocytosis time constants of GAT1 to 0.9 and 1.6 min. Furthermore, they investigated three different modulators of GAT1 trafficking which could be brought about via changes in the acutely recycling pool of GAT1 or on the endocytosis and exocytosis rates. Agonists of PKC were previously found to decrease the surface expression of GAT1, and this was accounted for by increases in endocytosis rate but does not change the acutely recycling pool. Hypertonic concentrations of sucrose were found to inhibit clathrin-dependent internalization not owing the effect to changes in the acutely recycling pool size. Finally, it was shown that Ca^{2+} depletion

caused a reduction in GAT1 surface expression due to diminished size of the acutely recycling pool (Wang and Quick, 2005).

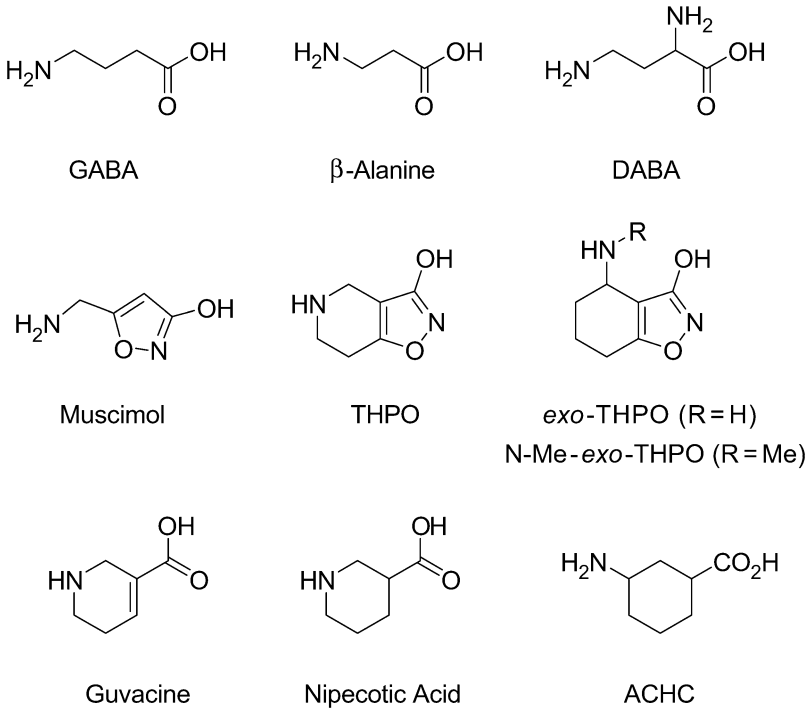
4 Pharmacology of GABA Transporters

A number of inhibitors, including substrates, exist that have been key tools in the elucidation of the significance of GABA transport and a brief overview of these compounds will be provided below. As mentioned above, compounds like DABA, ACHC, β-alanine, β-proline, and 3-hydroxy-5-aminovaleric acid have disclosed differences in the transport of GABA into glial and neuronal cells. These compounds are analogs of GABA in which the carbon chain of GABA is modified and/or substituted. Lately, 3-hydroxy-4-*N*-methylamino-4,5,6,7-tetrahydro-1,2-benzisoxazole (*N*-Me-*exo*-THPO) was reported as the most glia-selective inhibitor of GABA uptake yet. AU4 It was developed from muscimol, a constituent of the fly agaric mushroom *Amanita muscaria*, which can also act as an inhibitor of GABA uptake but has effects on the GABA receptors as well. The activity of muscimol arises from the ability of the 3-hydroxy-isoxazole moiety to act as a bioisosteric exchange for the carboxylic acid group in GABA. By using muscimol as lead structure, a series of related compounds was developed and among these THPO, a selective GABA uptake inhibitor was found (Krogsgaard-Larsen and Johnston, 1975; Krogsgaard-Larsen et al., 1975, 2000). This inhibitor enabled the discovery of the potent and selective inhibitors nipecotic acid and guvacine, which are both natural products. These two compounds have not only been valuable pharmacological tools but also very important lead structures for the development of highly potent GABA uptake inhibitors. The breakthrough was the addition of a lipophilic diaromatic side chain (Yunger et al., 1984; Ali et al., 1985), leading to the very potent compounds *N*-4,4-diphenylbut-3-en-1-yl-nipecotic acid (*N*-DPB-nipecotic acid/SKF89976A) and *N*-4,4-diphenylbut-3-en-1-yl-guvacine (*N*-DPB-guvacine/SKF100330A) that were nonsubstrate inhibitors and able to penetrate the AU5 blood–brain barrier (BBB) (Larsson et al., 1988). Following, an impressive number of compounds based on the nipecotic acid and guvacine scaffold with various lipophilic aromatic side chains have been synthesized and characterized (Pavia et al., 1992; Andersen et al., 1993, 1994, 1999, 2001a, b; Dhar et al., 1994; Knutsen et al., 1999). Among these, compounds like (*R*)-1-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-piperidinecarboxylic acid (tiagabine) and 1-(2-(((diphenylmethylene)amino)oxy)ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid (NNC-711) are highly GAT1 selective compounds. Most of the efforts in developing selective GABA uptake inhibitors were performed prior to the cloning of the individual GABA transporters, and highly specific and potent inhibitors are available only at GAT-1. This is probably a consequence of the predominant use of neuronal preparations for the characterization of compounds in which GAT1 is AU6 abundantly present as previously mentioned. Very recently, the development of (*R/S*)-4-[*N*-[1,1-bis(3-methyl-2-thienyl)but-1-en-4-yl]-*N*-methylamino]-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol (EF1502, Clausen et al., 2005) that selectively inhibits GAT1 and GAT2 without affecting GAT3 and GAT4 was reported, and further in vivo studies disclosed that GAT-2 could be an important therapeutic target (White et al., 2005). EF1502 is a structural hybrid of tiagabine and *N*-Me-*exo*-THPO. Initially, the *exo*-THPO series contained small alkyl substituents (Falch et al., 1999), but it was later expanded with lipophilic diaromatic side chains, leading to EF1502. Earlier 1-(3-(9*H*-carbazol-9-yl)-1-propyl)-4-(2-methoxyphenyl)-4-piperidinol (NNC 05–2090) has been reported as a selective GAT2 inhibitor; however, this compound also affects other transmitter systems (Thomsen et al., 1997). Also GAT3 and GAT4 can be simultaneously targeted with SNAP-5114 (Dhar et al., 1994), but effects at the other GABA transporter subtypes still persist. So whereas GAT1 is well characterized, there is still a need for potent and highly selective inhibitors of the other subtypes. The inhibitory activities of the above-mentioned GABA transport inhibitors are shown in ❯ *Table 18-2*, and a few important structures of GAT inhibitors are shown in ❯ *Figures 18-4* and ❯ *18-5*.

4.1 Anticonvulsant Activity of GABA Transport Inhibitors

Because the structurally restricted GABA analogs originally available (e.g., nipecotic acid, guvacine, and THPO) or later developed (*exo*-THPO) did not pass the BBB (Schousboe et al., 1986), the general strategy

Figure 18-4
Lead compounds used in the discovery of GABA transport inhibitors

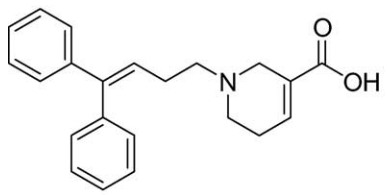


over the years relied on the identification of more lipophilic analogs (Yunger et al., 1984) or prodrugs (Krogsgaard-Larsen, 1981) as described above. *N*-DPB-nipecotic acid (SKF89976A) and *N*-DPB-guvacine (SKF100330A) displayed increased potency, the ability to penetrate the BBB, and were orally active when compared to their parent structure (Yunger et al., 1984; Ali et al., 1985). Moreover, they were shown not to be substrates for GABA transporters, although they acted as competitive inhibitors (Larsson et al., 1988). As previously mentioned, tiagabine and NNC-711 were identified from a mechanistic-based drug discovery program that targeted modification in the lipophilic aromatic side chain (Braestrup et al., 1990; Suzdak et al., 1992). Tiagabine was subsequently found to be a potent and systemically bioavailable anticonvulsant in rodent seizure and epilepsy models (Suzdak et al., 1992) and was developed and marketed as an add-on treatment for partial epilepsy (Suzdak and Jansen, 1995).

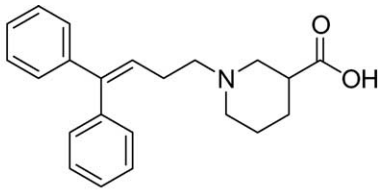
4.2 Glial Versus Neuronal Selective GAT1 Transport Inhibition

The ability of GABA transport inhibitors to prevent audiogenic seizures or chemically induced seizures was demonstrated a quarter of a century ago (Krogsgaard-Larsen, 1981; Croucher et al., 1983; Schousboe et al., 1983; Wood et al., 1983). As shown in ❯ *Table 18-3*, *exo*-THPO and selected *N*-substituted analogs displayed comparable anticonvulsant activity to the clinically active antiepileptic drug tiagabine when injected intracerebroventricularly into the brains of Frings audiogenic seizure-susceptible mice. Given that these compounds are considerably less potent than tiagabine as GABA transport inhibitors, it is somewhat surprising that they proved to be highly efficacious and unexpectedly potent as anticonvulsants when administered intracerebroventricularly (Gonsalves et al., 1989; White et al., 2002). Further pharmacological characterization of the unsubstituted as well as the *N*-methyl and *N*-ethyl substituted compounds

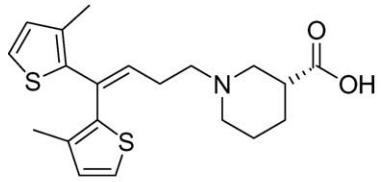
Figure 18-5
Various structures of more recent GABA transport inhibitors



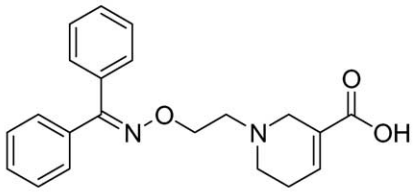
SKF100330A



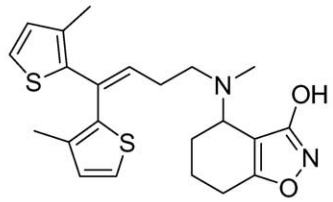
SKF89976A



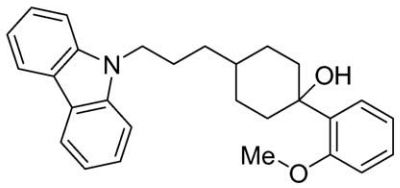
Tiagabine



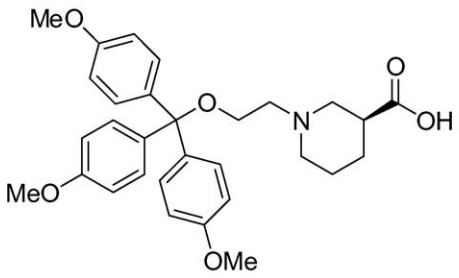
NNC-711



EF 1502



NNC 05-2090



SNAP-5114

disclosed that the anticonvulsive properties of these compounds when injected directly into the brain correlated well with the ability to inhibit glial GABA transport but not the corresponding neuronal transport (White et al., 2002). This finding supports the suggestion put forward two decades ago that inhibition of astrocytic GABA transport would likely lead to an increase in the pool of synaptic GABA and that selective inhibition of this transport system would facilitate reuptake into nerve endings leading to an

Table 18-3
Anticonvulsant activity of tiagabine and *exo*-THPO and its *N*-substituted analogs

	Anticonvulsant activity (nmol, i.c.v.)	
Compound	ED_{50}	90% CI
Tiagabine	22	11–36
exo-THPO	136	115–155
N-Methyl-*exo*-THPO	59	41–94
N-Ethyl-*exo*-THPO	155	88–255

From White et al. (2002) with permission

enhanced GABAergic tone protecting against epileptic seizures (Schousboe et al., 1983). Collectively, these findings demonstrate that if one is able to achieve therapeutic levels of a glial selective GABA transport inhibitor in the brain, it is likely to be as effective as tiagabine as an anticonvulsant.

4.3 Non-GAT1 Transport Inhibitors as Anticonvulsants

As mentioned above, the vast majority of GABA transport inhibitors primarily act on GAT1, which is preferentially located on neuronal elements with less expression on astrocytes. This observation has led to the suggestion that inhibitors of GABA transport mediated by transporters other than GAT1 may be interesting as anticonvulsants, especially those located extrasynaptically (see discussion below) (Dalby, 2003; Sarup et al., 2003b; Schousboe et al., 2004a). Indeed, several nonselective GABA transport inhibitors have been experimentally demonstrated to possess anticonvulsant activity (Dalby et al., 1997; Dalby, 2003; White et al., 2005). In this regard, EF1502 that is substituted with the side chain of tiagabine displays significant inhibitory effects on GAT2, which is notable given the high GAT1 selectivity of tiagabine. Considering that GABA has a lower K_m value at GAT2, EF1502 may be considered equipotent at GAT1 and GAT2.

Not surprisingly, EF1502 was found to possess a reasonably potent and broad-spectrum anticonvulsant profile when tested in a battery of animal seizure and epilepsy models following systemic administration (White et al., 2005). In this regard, EF1502 was found to possess a protective index (i.e., the ratio between median toxic and median effective doses) comparable to that of the selective GAT1 inhibitor tiagabine. Because EF1502 was equally active at both GAT1 and GAT2, isobolographic combination studies with EF1502, tiagabine, and another liphophilic GAT1-selective GABA-transport inhibitor LU-32–176B (*N*-[4,4-bis(4-fluorophenyl)-butyl]-4-amino-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol) were conducted to determine to what degree inhibition of GAT2 contributed to the anticonvulsant activity of EF1502. In these studies, EF1502 was found to exert a synergistic anticonvulsant activity when it was administered together with either tiagabine or LU-32–176B (White et al., 2005). More importantly, a less than additive interaction was observed when this combination was evaluated for behavioral toxicity. That the combination of EF1502 with tiagabine did not increase the behavioral toxicity of either drug alone is also of interest because it would suggest that selective inhibitors of the GAT2 transporter may offer some advantage over pure GAT1 selective inhibitors. The demonstration that inhibition of GAT2 by EF1502 likely contributes to its anticonvulsant activity supports the continued identification and development of GAT2-selective GABA transport inhibitors (Schousboe et al., 2004a; Clausen et al., 2005; White et al., 2005). Clearly, the development of a specific GAT2 inhibitor will be required before this hypothesis can be either confirmed or refuted.

5 Concluding Remarks

The GABA system obviously is of fundamental importance for the maintenance of brain function at all levels. Therefore, although the discussions concerning the pharmacology of GABA transporters have been

focused on their involvement in the control of seizure activity and epilepsy, it is likely that drugs acting on these transporters may be potential therapeutic candidates for other neurologic and psychiatric conditions believed to be associated with dysfunction of the GABA system, i.e., chronic pain, anxiety, sleep disorders, and others. Actually, several clinically effective drugs used for the treatment of these disorders do in fact act via interactions with entities of the GABA neurotransmission system such as receptor function. In this context, it may be pointed out that for inhibitors of the GABA transporters to have beneficial effects on GABA neurotransmission, their ability to change synaptic GABA levels needs to be translated into signaling through receptor interaction. With regard to the pharmacological interactions, in particular the non-GAT1 active inhibitors, it should be emphasized that since these transporters are located extrasynaptically on both neuronal and glial elements, it is likely that the action may involve extrasynaptic GABA receptors as suggested previously (Schousboe et al., 2004b; White et al., 2005). Of the $GABA_A$ receptor subunits, α6 AU7 and α4 associated with δ predominantly constitute the extrasynaptic receptor complexes inside and outside the cerebellum, respectively. It is shown that mice lacking the α6 subunit do not show the tonic component of $GABA_A$ receptor mediated inhibition of cerebellar granula cells (Mody, 2001). The development of THIP for the treatment of sleep disorders provides an excellent clinical demonstration supporting the role of extrasynaptic GABA receptors in the control of CNS function (Krogsgaard-Larsen et al., 2004).

Finally, it should be mentioned that in addition to direct interaction with the substrate binding site on GABA transporters, it is possible that modulating the expression of the transporters may represent an alternative therapeutic strategy for controlling CNS excitability. The fact that trafficking of these transporters between the cytoplasm and plasma membrane is rather dynamic (see above) opens the possibility for therapeutic intervention by compounds acting on protein kinases involved in the regulation of this process. However, such manipulation would be expected to be complicated by side effects due to the involvement of such kinases in a multitude of other functions. It may also be noted that endogenous proteins are likely to exist, which may directly regulate surface expression of GABA transporters. One such protein (GABA-CIP) was found to be secreted from cultured neurons (Nissen et al., 1992) and shown to increase the capacity for GABA transport in cerebellar astrocytes. Thus, one possible therapeutic strategy would be the development of molecules that decrease the expression and /or release of GABA-CIP. This would be expected to increase the level of synaptic and extrasynaptic GABA levels.

In summary, it is becoming increasingly clear that the regulation and trafficking of GABA transporters may have important implications for the treatment of a number of neurological disorders and diseases. As such, a greater understanding of the molecular biology, distribution, and factors that regulate their function will be critical for developing a new class of therapeutic agents that target this important regulator of CNS function.

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