Selective GABA Transporter Inhibitors Tiagabine and EF1502 Exhibit Mechanistic Differences in Their Ability to Modulate the Ataxia and Anticonvulsant Action of the Extrasynaptic GABA_A Receptor Agonist Gaboxadol

Karsten K. Madsen, Bjarke Ebert, Rasmus P. Clausen, Povl Krogsgaard-Larsen, Arne Schousboe, and H. Steve White

Departments of Pharmacology and Pharmacotherapy (K.K.M., A.S.) and Medicinal Chemistry (R.P.C., P.K.-L.), Faculty of Pharmaceutical Sciences, University of Copenhagen, Copenhagen, Denmark; Department of Electrophysiology, H. Lundbeck A/S, Valby, Denmark (B.E.); and Anticonvulsant Drug Development Program, Department of Pharmacology and Toxicology, University of Utah, Salt Lake City, Utah (H.S.W.)

Received January 20, 2011; accepted March 7, 2011

ABSTRACT

Modulation of the extracellular levels of GABA via inhibition of the synaptic GABA transporter GAT1 by the clinically effective and selective GAT1 inhibitor tiagabine [(*R*)-*N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]nipecotic acid; Gabitril] has proven to be an effective treatment strategy for focal seizures. Even though less is known about the therapeutic potential of other GABA transport inhibitors, previous investigations have demonstrated that *N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol (EF1502), which, like tiagabine, is inactive on GABA_A receptors, inhibits both GAT1 and the extrasynaptic GABA and betaine transporter BGT1, and exerts a synergistic anticonvulsant effect when tested in combination with tiagabine. In the present study, the anticonvulsant activity and motor impairment associated with systemic administration of ga-

boxadol (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol), which, at the doses used in this study (i.e., 1–5 mg/kg) selectively activates extrasynaptic α 4-containing GABA_A receptors, was determined alone and in combination with either tiagabine or EF1502 using Frings audiogenic seizure-susceptible and CF1 mice. EF1502, when administered in combination with gaboxadol, resulted in reduced anticonvulsant efficacy and Rotarod impairment associated with gaboxadol. In contrast, tiagabine, when administered in combination with gaboxadol, did not modify the anticonvulsant action of gaboxadol or reverse its Rotarod impairment. Taken together, these results highlight the mechanistic differences between tiagabine and EF1502 and support a functional role for BGT1 and extrasynaptic GABA_A receptors.

Introduction

The successful development of the selective inhibitor of GABA transporter subtype 1 (GAT1) tiagabine [(*R*)-*N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]nipecotic acid] as a clinically active drug for the treatment of focal seizures in humans has demonstrated that GABA transporters constitute an important molecular target for anticonvulsant drug devel-

opment (Nielsen et al., 1991). GABA transporters (GATs) facilitate the removal of GABA from the synapse into the presynaptic neurons and surrounding astrocytes. Reuptake of GABA into the presynaptic nerve endings serves to replenish the GABA pool for reutilization, whereas GABA taken up into astrocytes is metabolized and lost from the transmitter pool (Schousboe, 2003). Seizures, whether focal or generalized in origin, can result from an increase in excitatory neurotransmission or a decrease in inhibitory neurotransmission (Schousboe and White, 2009). GAT inhibitors have been shown, using in vivo microdialysis, to increase the extracellular concentration of GABA (Juhász et al., 1997; Dalby, 2000), which may lead to activation of GABA_A receptors and thus increase inhibitory neurotransmission within the cen-

This work was supported by the National Institutes of Health National Institutes of Neurological Disorders and Stroke [Contract N01-NS42359] (to H.S.W.); the Carlsberg Foundation [Grant 2009 01 0501] (to K.K.M.); and the Lundbeck Foundation [Grant R19-A2199] (to A.S.).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.111.179671.

ABBREVIATIONS: GAT1, GABA transporter subtype 1; GAT, GABA transporter; BGT1, betaine GABA transporter 1; GABA_A-R, GABA_A receptor; EF1502, *N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methylamino)-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol; LU-32-176, *N*-[4,4-bis(4-fluorophenyl)-butyl]-3-hydroxy-4-amino-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol; AGS, audiogenic seizure; TPE, time-to-peak effect; CI, confidence interval.

tral nervous system. This effect is thought to underlie the anticonvulsant action of tiagabine (Madsen et al., 2010).

Four GATs have been cloned from human, mouse, and rat tissues. However, because this has resulted in somewhat confusing nomenclature, that proposed by the HUGO Gene Nomenclature Committee will be used here, i.e., GAT1 (SLC6A1), BGT1 (SLC6A12), GAT2 (SLC6A13), and GAT3 (SLC6A11). Although an in-depth immunohistochemical study using knockout control animals remains to be undertaken, GATs are generally thought to be localized as follows: GAT1 is predominantly expressed on neurons at the synapse or to a minor extent on distal astrocytic processes throughout the mature brain (Borden, 1996; Conti et al., 1998). GAT3 is predominantly expressed on distal astrocytes, which are in direct contact with GABAergic neurons. GAT3 is highly expressed in retina, olfactory bulb, brainstem, and diencephalon but shows low levels of expression in hippocampus and cortex. In this regard GAT3 displays much more restricted localization than GAT1 (Minelli et al., 1996). GAT2 is found in the leptomeninges and the neonatal brain, and this transporter subtype is not believed to have a major effect on termination of GABAergic neurotransmission (Liu et al., 1993; Conti et al., 1999). BGT1 has been found in the hippocampus and cortex. In contrast to GAT1, BGT1 is not located close to GABAergic synapses but resides mainly in extrasynaptic regions (Borden et al., 1995; Zhu and Ong, 2004).

The distribution of GATs places them within the synapse as well as in the extrasynaptic region (Madsen et al., 2010). As a result, inhibitors of GATs indirectly modulate the interaction of GABA with two very different populations of GABA_A receptors (GABA_A-Rs), namely the synaptic and extrasynaptic members of this family. Synaptic GABA_A-Rs are typically composed of $\alpha\beta\gamma$ subunits, whereas extrasynaptically located GABA_A-Rs usually consist of δ subunits in complex with $\alpha 4$, $\alpha 5$, or $\alpha 6$ and β subunits (Nusser and Mody, 2002; Stell and Mody, 2003; Wei et al., 2003). Gaboxadol (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) has been established as a selective extrasynaptic GABA_A receptor agonist at concentrations that are pharmacologically relevant. It is important to note that gaboxadol is more efficacious but less potent than GABA at $\alpha 4$ -containing GABA_A-Rs (Störustovu and Ebert, 2006; Wafford and Ebert, 2006; Cremers and Ebert, 2007). Gaboxadol was developed as a sleep aid (Krosgaard-Larsen et al., 2004); however, for the purposes of the present study it also shows anticonvulsant properties and produces ataxia in mice (Christensen and Larsen, 1982; Chandra et al., 2006).

Proof of concept that GAT inhibitors mediate anticonvulsant activity in rodents has been firmly established (Nielsen et al., 1991; White et al., 1993, 2002, 2005; Madsen et al., 2009, 2010). Furthermore, it has been demonstrated that an inhibitor of BGT1 having no affinity for GABA_A-Rs, i.e., *N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-3-hydroxy-4-(methyldamino)-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol (EF1502) when administered in combination with inhibitors of GAT1 (i.e., tiagabine) and *N*-[4,4-bis(4-fluorophenyl)-butyl]-3-hydroxy-4-amino-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol (LU-32-176), results in a synergistic anticonvulsant effect in audiogenic seizure (AGS)-susceptible Frings mice (White et al., 2005). Because EF1502 also inhibits GAT1 (Clausen et al., 2005), additional experiments examining the combinatorial effect of the two GAT1

selective inhibitors tiagabine and LU-32-176 were performed. The results from the isobologram studies with these two GAT1 selective inhibitors suggested an additive anticonvulsant effect. These findings led to the conclusion that the ability of EF1502 to inhibit BGT1 contributes to the synergism observed with the GAT1-selective compounds, possibly by elevating the GABA concentration at extrasynaptic sites, which can thereby activate extrasynaptic GABA_A-Rs (White et al., 2005; Madsen et al., 2010).

In an effort to cast more light on the pharmacology of EF1502 versus that of tiagabine, a series of combination studies was designed to complement the previous studies (White et al., 2005; Madsen et al., 2009). The working hypothesis was developed to assess whether inhibition of GABA transport using either tiagabine or EF1502 would produce a sufficient increase in ambient GABA levels to displace the selective extrasynaptic GABA agonist gaboxadol and consequently counteract its anticonvulsant action and/or observed ataxia in mice. To address this question, combination studies were designed to investigate the anticonvulsant and ataxia interaction produced by the coadministration of gaboxadol with tiagabine or EF1502. This hypothesis is based on the demonstration that GABA transport inhibitors increase extracellular GABA concentrations (Juhász et al., 1997; Dalby, 2000) and that gaboxadol, at doses relevant to this study (i.e., 1–5 mg/kg), is a full agonist at extrasynaptic $\alpha 4$ -containing GABA_A-Rs but lacks activity at synaptic receptors. In contrast, GABA acts as a partial agonist at extrasynaptic receptors and as a full agonist at synaptic receptors (Chandra et al., 2006; Störustovu and Ebert, 2006; Wafford and Ebert, 2006; Cremers and Ebert, 2007). This important differentiation between gaboxadol and GABA provides the framework for the hypotheses described above.

Materials and Methods

Materials. EF1502 was synthesized as described previously (Clausen et al., 2005), tiagabine was generously provided by Cephalon Inc. (Frazer, PA), and gaboxadol was generously donated by H. Lundbeck A/S (Valby, Denmark).

Animals. Male albino CF1 mice (18–25 g) were obtained from Charles River Laboratories Inc. (Wilmington, MA). Male and female AGS-susceptible Frings mice were obtained from an in-house breeding facility at the University of Utah (Salt Lake City, UT). Mice were housed in a temperature-, humidity-, and light (on at 6:00 AM and off at 6:00 PM)-controlled facility. Animals were allowed free access to water and food (Harland Teklad 8640; Harland Laboratories, Indianapolis, IN) except when they were removed from their cages for testing. Furthermore, all mice were housed, fed, and handled in a manner consistent with the recommendations in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (publication 86-23, revised 1985; Institute of Laboratory Animal Resources, 1996). All experimental procedures were approved by the University of Utah Institutional Animal Care and Use Committee. Animals were euthanized in accordance with U.S. Public Health Service policies on the humane care of laboratory animals. All drugs were injected intraperitoneally.

Audiogenic Seizures. Seizures were evoked by placing individual Frings AGS-susceptible mice into a Plexiglas cylinder (diameter, 15 cm; height, 18 cm) fitted with an audio transducer (model AS-ZC; FET Research and Development, Salt Lake City, UT) and exposed to a sound stimulus of 110 db at 11 kHz for a duration of 20 s. Seizures are characterized by wild running and loss of righting reflex followed by all-limb clonus and subsequent tonic extension. Mice not displaying hind limb tonic extension were considered protected.

The test substances, EF1502, tiagabine, and gaboxadol were injected intraperitoneally and evaluated for antiseizure activity. First, the time-to-peak effect (TPE) was established by administration of a submaximal anticonvulsant dose to groups of four animals, which were tested at various time points (15, 30, 60, and 120 min) after drug administration. Thereafter, a dose-response curve was established at the predetermined TPE by giving animals injections ($n = 8$ mice/dose level) of varying doses of the test drug until at least two doses were established between the limits of 0 and 100% protection. The ED_{50} and the corresponding 95% confidence interval (CI) were calculated using data from the dose-response study, which was analyzed using probit analysis as described by Finney (1971). Gaboxadol was used in an isobologram study in combination with EF1502 or tiagabine to evaluate the resulting anticonvulsant interaction, which can be synergistic, additive, or antagonistic.

Isobologram Studies. Isobologram studies whereby combinations between drugs can be identified as being either additive, antagonistic, or synergistic were initially described by Löewe (1953) and further adapted by Tallarida (1992) and Tallarida et al. (1997). The method described here applies to drugs with parallelism of the slope function as obtained by probit analysis of the dose-response curve as described by Finney (1971). To verify parallelism of the slope functions between the different drugs a t test analysis was performed.

In brief, drug interaction studies were performed at three fixed-ratio combinations (3:1, 1:1, and 1:3), and isobolograms were constructed on the basis of the general equation of additivity, $a/A + b/B = 1$. A fixed-ratio combination of 1:3 means that the drug mixture ($a + b$) is composed of a ratio between one-fourth of the ED_{50} of drug A and three-fourths of the ED_{50} of drug B, respectively; thereby resulting in a combined theoretical additive $ED_{50, add}$ dose. The dose ratio between a and b is fixed, a dose-response curve is established, and the experimental $ED_{50, exp}$ is determined. The experimental $ED_{50, exp}$ is then compared with the theoretical additive $ED_{50, add}$ using a t test. An $ED_{50, exp}$ significantly higher, equal to, or lower than the $ED_{50, add}$ is considered antagonistic, additive, or synergistic, respectively. However, this statistical maneuver poses some problems. First, although the theoretical $ED_{50, add}$ dose is easily calculated as shown above, the variance term is another matter (see below). Second, because the data follow a normal distribution on the log scale, the $\log(ED_{50}) \pm S.E.M.[\log(ED_{50})]$ of both the theoretical and experimental fixed-ratio mixture needs to be obtained, before a t test can be performed. Equation 1 is used to convert the normal data to log data, thereby easily obtaining $\log(ED_{50, exp}) \pm S.E.M.[\log(ED_{50, exp})]$. To obtain the combined variance term for the theoretical $ED_{50, add}$ from both drugs A and B, eqs. 1 to 4 are used. Once the variance is obtained in eq. 4, eqs. 3 and 2 are used to approximate the $S.E.M.[\log(ED_{50, add})]$.

$$S.E.M.[\log(ED_{50})] = [\log(\text{upperCI}) - \log(ED_{50})]/1.96 \quad (1a)$$

$$S.E.M.[\log(ED_{50})] = [\log(ED_{50}) - \log(\text{lowerCI})]/1.96 \quad (1b)$$

$$S.E.M.(ED_{50}) = 2.3 \times (ED_{50}) \times S.E.M.[\log(ED_{50})] \quad (2)$$

$$V(ED_{50}) = S.E.M.(ED_{50})^2 \quad (3)$$

$$V(ED_{50, add}) = f_1^2 \times V(ED_{50, drug 1}) + (1 - f_1)^2 \times V(ED_{50, drug 2}) \quad (4)$$

If the 95% CI is not symmetric around the ED_{50} on the log scale, eqs. 1a and 1b are used to calculate the $S.E.M.(\log ED_{50, add})$ when antagonistic or synergistic interactions, respectively, are evaluated. The factor f_1 corresponds to the fraction of drug 1 in the mixture; hence, $f_1 = 1/4$ in the 1:3 fixed-ratio mixture. The degree of freedom of $V(ED_{50, add})$ is given by $N_{add} = N_1 + N_2 - 4$. Furthermore, as described above, the drugs were injected in a staggered fashion in such a manner that anticonvulsant testing in the Frings AGS-

susceptible mouse was conducted at the previously determined TPE for tiagabine and EF1502 (White et al., 2005).

Ataxia. In wild-type mice, 10 mg/kg gaboxadol induces ataxia, whereas in $\alpha 4$ GABA_A-R knockout mice, gaboxadol is without effect on motor function (Chandra et al., 2006). These results support the concept that gaboxadol is exerting its effects primarily through a direct activation of $\alpha 4$ -containing GABA_A receptors, which are thought to be located extrasynaptically (Nusser and Mody, 2002). In the present investigation, 5 mg/kg gaboxadol was found to produce a degree of ataxia comparable to that reported by Chandra et al. (2006). Ataxia was measured by placing CF1 mice on a fixed-speed Rotarod (6 rpm) and monitoring their performance for 180 s at various time points (15, 30, 45, 60, 75, and 90 min) after gaboxadol administration. The degree of ataxia was measured as the latency before the mouse fell off the rotating rod. Mice unable to complete a 180-s trial run on the Rotarod (6 rpm) when tested 3 h before the experiment were excluded from further study. Several combination studies to evaluate the interaction at this dose of gaboxadol, i.e., 5 mg/kg, and the GABA transport inhibitors tiagabine and EF1502 were performed. Tiagabine, at doses of 1 and 1.5 mg/kg, was combined with 5 mg/kg gaboxadol and similarly EF1502, at doses of 10 and 15 mg/kg, was combined with 5 mg/kg gaboxadol. Rotarod testing was performed at the time-to-peak effect for each of the drugs administered; i.e., 15, 30, and 60 min for gaboxadol, EF1502, and tiagabine, respectively. For example, tiagabine was administered 45 min before gaboxadol and the Rotarod test was performed 15 min later. It is important to note that no Rotarod impairment was observed at the doses of EF1502 and tiagabine tested (White et al., 2005). Recovery from ataxia induced by 5 mg/kg gaboxadol in the presence of EF1502 and tiagabine was evaluated using a two-way repeated-measures analysis of variance to control the values observed when gaboxadol was administered alone. Statistical analysis was performed using SigmaPlot 9.0 (Systat Software, Inc., San Jose, CA).

The combination studies to evaluate an interaction in the Rotarod test using CF1 mice and the seizure test using Frings AGS-susceptible mice were designed to test the hypothesis that elevation of ambient GABA levels would displace the more efficacious but less potent $\alpha 4$ GABA_A agonist gaboxadol from its binding site, thereby decreasing gaboxadol-induced motor impairment and altering its anticonvulsant efficacy.

Results

Acute Anticonvulsant Activity in Frings AGS-Susceptible Mice. Table 1 summarizes the anticonvulsant results obtained for gaboxadol, tiagabine, and EF1502 in AGS-susceptible Frings mice. All three compounds were found to produce an anticonvulsant effect in this mouse model of sensory evoked seizures.

Isobologram Studies. The experimental and theoretical results obtained from several fixed-dose combination studies are summarized in Table 2. The calculations of the theoretical $ED_{50, add} \pm S.E.M.$ and experimental $ED_{50, exp} \pm S.E.M.$

TABLE 1
Anticonvulsant efficacy of gaboxadol, tiagabine, and EF1502 in Frings AGS-susceptible mice

All anticonvulsant studies were conducted as described under *Materials and Methods* at the TPE determined for each of the drugs tested. The ED_{50} and 95% CI were calculated by probit analysis. n represents the total number of animals used to obtain the dose-response curve; in general, eight mice were used per dose level.

Compound	TPE	ED_{50}	95% CI	n
	min		mg/kg	
Gaboxadol	15	4.2	2.45–5.87	40
Tiagabine	60	0.52	0.34–0.70	54
EF1502	30	4.85	3.61–6.25	39

TABLE 2

Theoretical and experimental isobologram results obtained from combined fixed-dose mixtures of gaboxadol and tiagabine or gaboxadol and EF1502 in the Frings audiogenic seizure-susceptible mouse. Theoretical additive dose and experimental determined dose are represented by $ED_{50, add}$ and $ED_{50, exp}$, respectively. S.E.M. values are calculated as described under *Materials and Methods*.

Mixture	$ED_{50, add} \pm S.E.M.$	$ED_{50, exp} \pm S.E.M.$	<i>n</i>
	<i>mg/kg</i>		
Gaboxadol-tiagabine, 1:3	1.44 ± 0.19	1.78 ± 0.35	34
Gaboxadol-tiagabine, 1:1	2.36 ± 0.36	3.32 ± 0.47	33
Gaboxadol-tiagabine, 3:1	3.28 ± 0.54	4.47 ± 0.45	33
Gaboxadol-EF1502, 1:3	4.68 ± 0.50	$9.84 \pm 2.67^{***}$	32
Gaboxadol-EF1502, 1:1	4.52 ± 0.48	$14.68 \pm 1.40^{***}$	32
Gaboxadol-EF1502, 3:1	4.52 ± 0.48	N.A.	N.A.

N.A., not available because establishment of the ED_{50} value was impossible because of extensive antagonism.

*** Significant antagonistic anticonvulsant effect ($p < 0.001$) using Student's *t* test.

are described above. As explained under *Materials and Methods*, the slope function of the drugs in the mixture must be parallel for proper isobolographic analysis. In the present study, the slope functions of gaboxadol, tiagabine, and EF1502 were not significantly different from each other and thus met the criteria for isobolographic analysis (data not shown). As summarized in Table 2 and shown graphically in Fig. 1A, the combination of gaboxadol and tiagabine exhibited an additive interaction, whereas the combination of gaboxadol and EF1502 (Fig. 1B) displayed an antagonistic interaction.

Ataxia. At a dose of 5 mg/kg, gaboxadol displayed a time-dependent motor impairment in the Rotarod test (Fig. 2, A and B). On the basis of the hypothesis that elevation of ambient GABA levels would lead to the displacement of gaboxadol from its binding site at the $\alpha 4$ GABA_A-R, combination studies were conducted with the GAT1 selective inhibitor tiagabine and the mixed GAT1/BGT1 inhibitor EF1502. As shown in Fig. 2A, tiagabine at two different doses (1 and 1.5 mg/kg) did not significantly ameliorate the motor impairment induced by gaboxadol. Contrary to the lack of effect of tiagabine, EF1502 at two different doses (10 and 15 mg/kg) significantly ($p < 0.05$) attenuated the motor impairment associated with gaboxadol (Fig. 2B).

Discussion

Previous investigations have shown a synergistic anticonvulsant action between the GAT1 selective inhibitor tiagabine and the GAT1/BGT1 inhibitor EF1502 (White et al., 2005; Madsen et al., 2009). These results provided the basis for suggesting that BGT1 plays a role in controlling central nervous system excitability. As discussed above, GAT1 is located at synaptic sites, whereas BGT1 is thought to reside primarily at extrasynaptic sites (Madsen et al., 2010). The differences in their anatomical localization make these two GABA transporters ideally suited to modulate GABA-mediated inhibition throughout the brain. Previous microdialysis studies have clearly demonstrated that GABA transport inhibitors elevate extracellular GABA concentrations (Juhász et al., 1997; Dalby, 2000). Moreover, studies by Störustovu and Ebert (2006) demonstrated that gaboxadol acts as a "super" agonist at $\alpha 4$ -containing GABA_A receptors. Activation of these receptors, which are presumed to be localized to extrasynaptic sites (Nusser and Mody, 2002), is thought to

Fig. 1. Graphical representation of the isobologram at the ED_{50} level. The theoretical line of additivity connects diagonally the ED_{50} value of gaboxadol and tiagabine (A) or gaboxadol and EF1502 (B) on both axes. On the line of additivity the theoretical $ED_{50, add}$ values of the three fixed-ratio combinations (1:3, 1:1, and 3:1) are plotted with their corresponding S.E.M. (●). In addition, at the same fixed-ratio combinations, the experimentally derived $ED_{50, exp} \pm S.E.M.$ values are presented (□). ***, statistically significant antagonistic anticonvulsant effect as observed with the gaboxadol and EF1502 combination ($p < 0.001$). #, dose-response curve for the 3:1 combination could not be established unless very high amounts of compounds were used, thereby suggesting an antagonistic interaction with this dose combination.

underlie the mechanism through which gaboxadol exerts its soporific action (Krogsgaard-Larsen et al., 2004). In this context, it should be noted that the ataxia induced by gaboxadol is completely ameliorated in knockout mice lacking expression of the $\alpha 4$ subunit (Chandra et al., 2006).

With this new knowledge regarding the molecular target and mechanism of action of gaboxadol in hand, a new approach was undertaken to differentiate the pharmacological profile of tiagabine from that of EF1502. The results from the initial study demonstrated that gaboxadol was anticonvulsant in Frings AGS-susceptible mice and that it was capable

Fig. 2. Effect of tiagabine (A) and EF-1502 (B) on gaboxadol-induced ataxia. Gaboxadol (5 mg/kg i.p.) produced a time-dependent ataxia (▲; A and B). A, effect of coadministration of 1 mg/kg (○) and 1.5 mg/kg (●) tiagabine on gaboxadol-induced Rotarod performance. B, effect of coadministration of 10 (□) and 15 mg/kg (■) EF1502 on gaboxadol-induced Rotarod impairment. At both doses tested, EF1502 significantly diminished the ataxia associated with gaboxadol ($p < 0.05$ by two-way analysis of variance repeated-measures analysis with Fisher's least significant difference post hoc test). Gaboxadol, EF1502, and tiagabine were administered at time (t) 0, $t - 15$, and $t - 45$ min, respectively. $n = 12$ mice for all studies shown in A and B.

of producing motor impairment at doses that were comparable to those reported previously (Cremers and Ebert, 2007). These results indirectly confirmed the presence of $\alpha 4$ -containing GABA_A receptors and provided the rationale for the subsequent combination studies. These studies tested the hypothesis that elevation of extracellular GABA by the GABA transport inhibitors tiagabine and EF1502 (Juhász et al., 1997; Dalby, 2000) would be sufficient to displace gaboxadol from its binding site and reverse its anticonvulsant action and ability to induce ataxia in AGS-susceptible Frings mice and CF1 mice, respectively.

Although not directly assessed in this study, the *in vivo* results obtained in the combination studies with EF1502 and gaboxadol support the conclusion that ambient extrasynaptic GABA levels were elevated. This conclusion is based on the demonstration that the *in vivo* action of gaboxadol was antagonized by EF1502. In contrast, tiagabine did not display an antagonist interaction with gaboxadol under the conditions in which it was studied. The differences between EF1502 and tiagabine can be explained on the basis of the anatomical location of their respective targets of action. For

example, GAT1 is localized close to the synapse, whereas BGT1 is localized extrasynaptically (for review, see Madsen et al., 2010). Thus, tiagabine, which is a highly selective GAT1 inhibitor, would be expected to lead to a preferential increase in synaptic GABA levels over extrasynaptic GABA levels, whereas EF1502 by virtue of its ability to inhibit both GAT1 and BGT1 would be expected to increase both synaptic and extrasynaptic GABA concentrations. This does not suggest that there would not be some spillover of GABA to extrasynaptic sites after TGB administration; however, it would probably be minimal relative to that produced by the GAT1/BGT1 inhibitor EF1502. Unfortunately, it would be extremely difficult from a technical perspective to measure where the spillover is occurring, e.g., synaptic versus extrasynaptic, after TGB and EF1502 administration. Thus, we are left to interpret the behavioral results in light of the anatomical studies that have been conducted, and although the interpretation is open to criticism, the results support the current working hypothesis and emphasize the need for additional investigation.

In summary, the approach used in this study clearly highlights the differences in the mechanistic and functional profile of tiagabine and EF1502 and their respective molecular targets. Nonetheless, any final conclusion regarding the overall role of extrasynaptic GABA transporters such as BGT1 will have to await the design and development of highly selective inhibitors that target these transporters.

Acknowledgments

We cordially thank Klaus K. Holst (Department of Biostatistics, University of Copenhagen) for help in designing the statistics for interpretation of isobologram results. We also thank Tim Pruess for his expert technical assistance.

Authorship Contributions

Participated in research design: Madsen, Ebert, Schousboe, and White.

Conducted experiments: Madsen.

Contributed new reagents or analytic tools: Ebert, Clausen, and Krogsgaard-Larsen.

Performed data analysis: Madsen.

Wrote or contributed to the writing of the manuscript: Madsen, Schousboe, and White.

References

- Borden LA (1996) GABA transporter heterogeneity: pharmacology and cellular localization. *Neurochem Int* 29:335–356.
- Borden LA, Smith KE, Gustafson EL, Branchek TA, and Weinshank RL (1995) Cloning and expression of a betaine/GABA transporter from human brain. *J Neurochem* 64:977–984.
- Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF, Spigelman I, Houser CR, Olsen RW, Harrison NL, et al. (2006) GABA_A receptor $\alpha 4$ subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. *Proc Natl Acad Sci USA* 103:15230–15235.
- Christensen AV and Larsen JJ (1982) Antinociceptive and anticonvulsive effect of THIP, a pure GABA agonist. *Pol J Pharmacol Pharm* 34:127–134.
- Clausen RP, Moltzen EK, Perregaard J, Lenz SM, Sanchez C, Falch E, Frølund B, Bolvig T, Sarup A, Larsson OM, et al. (2005) Selective inhibitors of GABA uptake: synthesis and molecular pharmacology of 4-N-methylamino-4,5,6,7-tetrahydrobenzo[d]isoxazol-3-ol analogues. *Bioorg Med Chem* 13:895–908.
- Conti F, Melone M, De Biasi S, Minelli A, Brecha NC, and Ducati A (1998) Neuronal and glial localization of GAT-1, a high-affinity gamma-aminobutyric acid plasma membrane transporter, in human cerebral cortex: with a note on its distribution in monkey cortex. *J Comp Neurol* 396:51–63.
- Conti F, Zuccarello LV, Barbaresi P, Minelli A, Brecha NC, and Melone M (1999) Neuronal, glial, and epithelial localization of γ -aminobutyric acid transporter 2, a high-affinity γ -aminobutyric acid plasma membrane transporter, in the cerebral cortex and neighboring structures. *J Comp Neurol* 409:482–494.
- Cremers T and Ebert B (2007) Plasma and CNS concentrations of Gaboxadol in rats following subcutaneous administration. *Eur J Pharmacol* 562:47–52.

- Dalby NO (2000) GABA-level increasing and anticonvulsant effects of three different GABA uptake inhibitors. *Neuropharmacology* **39**:2399–2407.
- Finney DJ (1971) *Probit Analysis*. Cambridge University Press, London.
- Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals* 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington DC.
- Juhász G, Kékesi KA, Nyitrai G, Dobolyi A, Krogsgaard-Larsen P, and Schousboe A (1997) Differential effects of nipecotic acid and 4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol on extracellular γ -aminobutyrate levels in rat thalamus. *Eur J Pharmacol* **331**:139–144.
- Krogsgaard-Larsen P, Frølund B, Liljefors T, and Ebert B (2004) GABA(A) agonists and partial agonists: THIP (Gaboxadol) as a non-opioid analgesic and a novel type of hypnotic. *Biochem Pharmacol* **68**:1573–1580.
- Liu QR, López-Corcuera B, Mandiyan S, Nelson H, and Nelson N (1993) Molecular characterization of four pharmacologically distinct gamma-aminobutyric acid transporters in mouse brain [corrected]. *J Biol Chem* **268**:2106–2112.
- LOEWE S (1953) The problem of synergism and antagonism of combined drugs. *Arzneimittelforschung* **3**:285–290.
- Madsen KK, Clausen RP, Larsson OM, Krogsgaard-Larsen P, Schousboe A, and White HS (2009) Synaptic and extrasynaptic GABA transporters as targets for anti-epileptic drugs. *J Neurochem* **109** (Suppl 1):139–144.
- Madsen KK, White HS, and Schousboe A (2010) Neuronal and non-neuronal GABA transporters as targets for antiepileptic drugs. *Pharmacol Ther* **125**:394–401.
- Minelli A, DeBiasi S, Brecha NC, Zuccarello LV, and Conti F (1996) GAT-3, a high-affinity GABA plasma membrane transporter, is localized to astrocytic processes, and it is not confined to the vicinity of GABAergic synapses in the cerebral cortex. *J Neurosci* **16**:6255–6264.
- Nielsen EB, Suzdak PD, Andersen KE, Knutsen LJ, Sonnewald U, and Braestrup C (1991) Characterization of tiagabine (NO-328), a new potent and selective GABA uptake inhibitor. *Eur J Pharmacol* **196**:257–266.
- Nusser Z and Mody I (2002) Selective modulation of tonic and phasic inhibitions in dentate gyrus granule cells. *J Neurophysiol* **87**:2624–2628.
- Schousboe A (2003) Role of astrocytes in the maintenance and modulation of glutamatergic and GABAergic neurotransmission. *Neurochem Res* **28**:347–352.
- Schousboe A and White HS (2009) Glial modulation of excitability via glutamate and GABA transporters, in *Encyclopedia of Basic Epilepsy Research* (Schwartzkroin PA ed) pp 397–401, Academic Press, Oxford.
- Stell B and Mody I (2003) A tale of timing and transport. *Neuron* **39**:729–730.
- Stórustovu SI and Ebert B (2006) Pharmacological characterization of agonists at δ -containing GABA_A receptors: Functional selectivity for extrasynaptic receptors is dependent on the absence of γ_2 . *J Pharmacol Exp Ther* **316**:1351–1359.
- Tallarida RJ (1992) Statistical analysis of drug combinations for synergism. *Pain* **49**:93–97.
- Tallarida RJ, Stone DJ Jr, and Raffa RB (1997) Efficient designs for studying synergistic drug combinations. *Life Sci* **61**:PL 417–PL 425.
- Wafford KA and Ebert B (2006) Gaboxadol—a new awakening in sleep. *Curr Opin Pharmacol* **6**:30–36.
- Wei W, Zhang N, Peng Z, Houser CR, and Mody I (2003) Perisynaptic localization of δ subunit-containing GABA_A receptors and their activation by GABA spillover in the mouse dentate gyrus. *J Neurosci* **23**:10650–10661.
- White HS, Hunt J, Wolf HH, Swinyard EA, Falch E, Krogsgaard-Larsen P, and Schousboe A (1993) Anticonvulsant activity of the gamma-aminobutyric acid uptake inhibitor N-4,4-diphenyl-3-butenyl-4,5,6,7-tetrahydroisoxazolo[4,5-c]pyridin-3-ol. *Eur J Pharmacol* **236**:147–149.
- White HS, Sarup A, Bolvig T, Kristensen AS, Petersen G, Nelson N, Pickering DS, Larsson OM, Frølund B, Krogsgaard-Larsen P, et al. (2002) Correlation between anticonvulsant activity and inhibitory action on glial γ -aminobutyric acid uptake of the highly selective mouse γ -aminobutyric acid transporter 1 inhibitor 3-hydroxy-4-amino-4,5,6,7-tetrahydro-1,2-benzisoxazole and its N-alkylated analogs. *J Pharmacol Exp Ther* **302**:636–644.
- White HS, Watson WP, Hansen SL, Slough S, Perregaard J, Sarup A, Bolvig T, Petersen G, Larsson OM, Clausen RP, et al. (2005) First demonstration of a functional role for central nervous system betaine/ γ -aminobutyric acid transporter (mGAT2) based on synergistic anticonvulsant action among inhibitors of mGAT1 and mGAT2. *J Pharmacol Exp Ther* **312**:866–874.
- Zhu XM and Ong WY (2004) A light and electron microscopic study of betaine/GABA transporter distribution in the monkey cerebral neocortex and hippocampus. *J Neurocytol* **33**:233–240.

Address correspondence to: Dr. H. Steve White, Department of Pharmacology and Toxicology, University of Utah, 417 Wakara Way, Suite 3211, Salt Lake City, Utah 84108. E-mail: swhite@hsc.utah.edu