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Synergy between retigabine and GABA in modulating the convulsant site of the $GABA_A$ receptor complex

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Abstract

The molecular mechanism underlying the activity of the novel antiepileptic drug retigabine is not yet fully understood. The aim of this study was to investigate whether retigabine interacts directly with the $GABA_A$ receptor complex (γ-aminobutyric acid). Receptor-binding assays were conducted using rat brain membranes. [^{3}H]-*t*-Butyl-bicyclo-orthobenzoate ([^{3}H]TBOB) was used as a tracer ligand. We determined the effects of GABA and retigabine in the presence of several concentrations of GABA on the binding of [^{3}H]TBOB. GABA inhibited [^{3}H]TBOB binding with an EC_{50} of 4.8 μM. In the absence of GABA, retigabine inhibited [^{3}H]TBOB with an EC_{50} of 124 μM and an EC_{50} of 42 μM in the presence of 2.5 μM GABA. Isobolic analysis revealed that retigabine acts in synergy with GABA in displacing [^{3}H]TBOB. This synergy could be quantified by a molecular model in which GABA and retigabine both allosterically displace [^{3}H]TBOB, and retigabine allosterically enhances the binding of GABA and vice versa with a factor of 4. In summary, we found that retigabine does indeed interact with a site on the $GABA_A$ receptor complex, and this site is positively allosterically coupled with the GABA site. This GABA-positive effect may well contribute to the clinical anticonvulsive effects of retigabine.

Keywords: Retigabine; $GABA_A$ receptor complex; TBOB; Synergy; Isobole method; Molecular model

1. Introduction

Retigabine, (D-23129) [*N*-(2-amino-4-(4-flurobenzylamino)phenyl)carbamid acid ethyl ester], is a new antiepileptic drug. It is effective in a variety of animal models for convulsions (Rostock et al., 1996). At present the drug is in clinical development (Ferron et al., 2002).

The molecular mechanism underlying the activity of retigabine is not yet fully understood. In vitro experiments showed that retigabine activates K^+ channels (Main et al., 2000; Rundfeldt, 1997; Schroder et al., 2001; Tatulian et al., 2001; Wickenden et al., 2000), and thus suppresses neuronal firing (Rogawski, 2000). In addition, it was suggested that an activation of GABA-ergic (γ-aminobutyric acid-ergic) inhibition adds to the clinical anticonvulsive effects of retigabine: an enhancement of GABA-induced inhibitory postsynaptic Cl^- currents was reported (Otto et al., 2002; Rundfeldt and Netzer, 2000). This GABA-ergic mechanism includes an increase in GABA concentration by an increase of the synthesis of GABA (Kapetanovic et al., 1995) and by an inhibition of its metabolism (Sills et al., 2000). In addition, the GABA-ergic effect is suggested to result from a direct interaction of retigabine with a site on the $GABA_A$ receptor complex (Otto at al., 2002). $GABA_A$ receptors are ligand-gated chloride ion channels. The $GABA_A$ receptor complex comprises binding sites for a variety of compounds, such as benzodiazepines, barbiturates, neuroactive steroids and general anaesthetics; furthermore, a picrotoxin-sensitive convulsant site is present on the complex (review: Mehta and Ticku, 1999). A number of these binding sites are allosterically coupled, resulting in a network of interactions, ultimately regulating the permeability of the Cl^- channel (Mehta and Ticku, 1999; Korpi et al., 2002). The functional state of the channel can be assayed by measuring the amount of ligand binding to the picrotoxin-sensitive convulsant site (Havoundjian et al., 1986; Maksay, 1996; Wha Bin Im and Blakeman, 1991; Korpi et al., 2002). We can therefore measure the effects of allosteric interactions that modulate GABA-ergic neurotransmission using receptor-binding assays on the picrotoxin-sensitive convulsant site. [^{3}H]-*t*-Butyl-bicyclo-orthobenzoate ([^{3}H]TBOB) can be

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used as a tracer ligand for the picrotoxin-sensitive convulsant site (Lawrence et al., 1985; Van Rijn et al., 1990).

The aim of the present study was to investigate whether retigabine interacts directly with the $GABA_A$ receptor complex and, if so, whether it interacts with the binding of GABA. In vitro receptor binding assays were conducted using well-washed rat brain membranes. We determined the effects of GABA alone and of retigabine in the presence of several concentrations of GABA on the binding of [^{3}H]TBOB. The experimental data were described in two different ways. First, the sigmoid-E_{max} model was fitted to the data in order to describe the results in terms of additivity using the isobole method (Berenbaum, 1989; Greco et al., 1995; Loewe, 1953; Tallarida, 1992), which allows qualitative conclusions (Greco et al., 1995). Next, the allosteric three-ligand molecular model, the cubic ternary complex (Kenakin, 1997), was fitted to the data, which allowed us to describe the observed interaction in quantitative terms as well (Van Rijn et al., 1999).

2. Materials and methods

2.1. Preparation of the tissue

This study was performed in accordance with the guidelines of the European Community for the use of experimental animals. Approval of the local ethical committee for animal studies was obtained. Forebrains of Wistar rats [body weight 350 ± 50 g (mean ± S.D.)] were used. The brains were homogenised in 9 volumes of 0.32 M sucrose at 0 °C with a Teflon-glass homogeniser. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C. The supernatant was decanted and centrifuged at 48,000 × *g* for 30 min at 4 °C. The pellets were washed two times by suspension in 50 mM sodium–potassium–phosphate buffer, pH 7.4, containing 500 mM NaCl (assay buffer), and centrifugation at 48,000 × *g* for 10 min at 4 °C. The pellets were frozen, thawed and washed three times in order to remove endogenous GABA. The pellets were stored at −80 °C until assay. Before assay, the pellets were washed once.

2.2. Assays

The pellets were homogenised in assay buffer: the tissue concentration in the incubation medium was 12.5 mg tissue wet weight/ml. Into glass tubes we added consecutively 25 μl of [^{3}H]TBOB and either drugs or buffer in volumes of 25 μl till a volume of 100 μl. The final concentration of [^{3}H]TBOB was 8 nM. The incubation was started by adding 200 μl of tissue homogenate. Incubations were performed at 25 °C, lasted 90 min and were terminated by adding 3 ml ice-cold buffer to the tubes and rapid filtration of the mixture. The filters were washed two times with 3 ml cold assay buffer. Radioactivity retained in the filters was counted by liquid scintillation spectrometry. Specific [^{3}H]TBOB binding was defined as total binding minus the remaining binding in the presence of 100 μM picrotoxin. Specific binding was 65–70% of total binding at 8 nM [^{3}H]TBOB.

[^{3}H]TBOB displacement curves were constructed for GABA and for retigabine in the absence of exogenous GABA and in the presence of 0.4, 1.0 and 2.5 μM added GABA.

2.3. Chemicals

GABA and picrotoxin were obtained from Sigma-Aldrich Chemie. [^{3}H]TBOB was obtained from Amersham Biosciences. The specific activity was 18 Ci/mmol (batch 28). Retigabine was a gift from Viatris, Frankfurt am Main. Retigabine was dissolved in 1 N HCl and diluted 1000 times with buffer during incubation.

2.4. Data analysis

2.4.1. Description of the displacement curves

The right-hand side of the sigmoid-E_{max} Eq. (1) was fitted to the data in order to describe the displacement curves.

$$E_{drug} = \frac{E_{max}}{1 + \left[\frac{EC_{50}}{[drug]}\right]^H} \quad (1)$$

In Eq. (1), [drug] is the concentration of test drug in mol/l. E_{max} is the experimentally determined binding of [^{3}H]TBOB in the absence of test drug, representing 100% binding. E_{drug} is the experimentally determined binding of [^{3}H]TBOB in the presence of the test drug, expressed as a percentage of E_{max} binding. EC_{50} is the concentration of the drug that leaves 50% binding of [^{3}H]TBOB, and H is the Hill coefficient. EC_{50} and H were estimated by nonlinear regression analysis.

2.4.2. Isobolic analysis of the data

An isobologram was constructed for the EC_{35}, the concentration of test drug that leaves 35% binding of [^{3}H]TBOB. Instead of 50%, this 35% effect point was chosen since this point is on the steep part of all the measured dose–effect curves. EC_{35} parameters were estimated using the rewritten sigmoid-E_{max} model (Eq. (1A)).

$$E_{drug} = \frac{E_{max}}{1 + \left[\frac{E_{max} - E_{35}}{E_{35}}\right]\left[\frac{EC_{35}}{[drug]}\right]^H} \quad (1A)$$

In an isobologram, the concentration of one drug (GABA) is represented on the abscissa; the concentration of the other drug (retigabine) is represented on the ordinate.

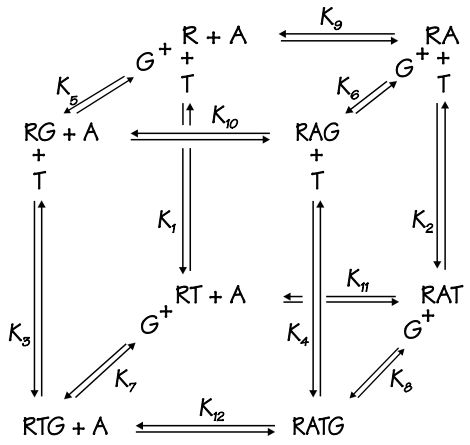

Fig. 1. The allosteric three-ligand molecular model for interactions between GABA, retigabine and [^{3}H]TBOB. The model reflects a set of bimolecular reversible reactions, in which R is a receptor site. Three receptor sites are assumed for three ligands: a site for GABA (G), a site for retigabine (A) and a site for [^{3}H]TBOB (T). K_i's are dissociation constants. All three sites are allosterically coupled. The experimental data are described when GABA displaces allosterically [^{3}H]TBOB such that $K_3 = \delta K_1$ with $\delta \gg 1$; retigabine displaces allosterically [^{3}H]TBOB such that $K_2 = \beta K_1$ with $\beta \gg 1$. The change in K_d of TBOB by binding of both GABA and retigabine is described by ε such that $K_4 = \varepsilon K_3 = \varepsilon\delta K_1$ with $\varepsilon \gg 1$. Retigabine enhances allosterically the binding of GABA and vice versa such that $K_6 = \gamma K_5$ and $K_{10} = \gamma K_9$ with $\gamma < 1$.

Each plotted point in the graph represents a pair of concentrations of the two drugs needed for 35% binding when added in combination. The straight line that connects the two plotted points of the pure single drugs is the theoretical isobole. If the experimentally determined data points lie on this straight line, then the drug effects are purely additive (no interaction). If the points lie below this line, then there is supra-additivity (synergy), and if they lie above this line, then there is subadditivity (antagonism). This can be made understandable by calculating the total drug load (Deckers et al., 1997) as follows: drug load=(added GABA)/($EC_{35\ \text{pure GABA}}$)+($EC_{35\ \text{retigabine in combination with GABA}}$)/($EC_{35\ \text{pure retigabine}}$) (Berenbaum, 1989; Tallarida, 1992). If the total drug load is below unity, then there is synergy.

2.4.3. Molecular modelling of the data

A molecular model for allosteric interactions between retigabine, GABA and [^{3}H]TBOB is depicted in Fig. 1. The model reflects a set of reversible bimolecular reactions, in which R is a receptor site. Three receptor sites are assumed: one site for retigabine (A), one for GABA (G) and one for [^{3}H]TBOB (T). The empty receptor complex is abbreviated as R. Eq. (2) is the overall expression describing the allosteric three-ligand molecular model labelled with [^{3}H]TBOB. This model is described by Kenakin (1997) and an extension of this model is described by Van Rijn et al. (1999). The derivation of this equation is given in Appendix A.

$$\frac{[\text{Bound}_{\text{tot}}]}{[\text{R}_{\text{tot}}]} = \frac{\frac{[\text{T}]}{K_1}\left(1 + \frac{[\text{A}]}{\beta K_9} + \frac{[\text{G}]}{\delta K_5} + \frac{[\text{A}][\text{G}]}{\gamma\delta\varepsilon K_5 K_9}\right)}{1 + \frac{[\text{A}]}{K_9} + \frac{[\text{G}]}{K_5} + \frac{[\text{A}][\text{G}]}{\gamma K_5 K_9} + \left(1 + \frac{[\text{A}]}{\beta K_9} + \frac{[\text{G}]}{\delta K_5} + \frac{[\text{A}][\text{G}]}{\gamma\delta\varepsilon K_5 K_9}\right)} \quad (2)$$

In which [T] is the concentration [^{3}H]TBOB, [A] is the concentration of retigabine and [G] is the concentration of GABA. The parameters K_1, K_5, K_9, β, γ, δ and ε were to be estimated. The equation was fitted to the data using non-linear regression analysis for which the software program Prism 3.1 (GraphPad Software) was used.

3. Results

3.1. Fitting the sigmoid-E_{max} Eq. (1) to the data

Parameter estimates are given in Table 1, data points and best fits are given in Fig. 2. GABA displaced [^{3}H]TBOB with an EC_{50} in the low micromolar range. Retigabine displaced [^{3}H]TBOB both in the absence and in the presence of GABA. The effective concentrations were in the high micromolar range. The ED_{50} of retigabine decreased with increasing concentration of GABA. The Hill coefficients were not significantly different from unity.

Table 1
Parameter estimates of the sigmoid-E_{max} curve fitted to the experimental data

Drug	Added GABA (μM)	EC_{50}		EC_{35}		Total drug load, 35% binding		Hill		n
		(μM)	(95% CI)	(μM)	(95% CI)	Fraction of pure drugs	(95% CI)	Number	(95% CI)	
GABA	–	4.83	(3.64, 6.42)	8.67	(6.68, 11.3)	1.0	(0.77, 1.30)	1.06	(0.82, 1.30)	6
Retigabine	0	124	(103, 149)	216	(171, 274)	1.0	(0.79, 1.26)	1.11	(0.85, 1.37)	6
Retigabine	0.4	79.1	(65.8, 94.9)	140	(115, 169)	0.69	(0.57, 0.84)	0.92	(0.73, 1.07)	4
Retigabine	1.0	57.4	(42.7, 77.2)	81.9	(63.1, 106)	0.49	(0.38, 0.63)	0.96	(0.68, 1.23)	4
Retigabine	2.5	41.5	(32.4, 53.3)	38.2	(30.9, 47.3)	0.47	(0.37, 0.58)	1.18	(0.86, 1.49)	4

The sigmoid-E_{max} Eq. (1) (EC_{50} and Hill parameter) and Eq. (1A) (EC_{35}) were fitted to the experimental data. The parameter estimates and the 95% confidence intervals (CI) of the fits are given. When the CIs do not overlap, the values are significantly different. Retigabine displaced [^{3}H]TBOB in the absence and in the presence of added GABA. The EC_{50} of retigabine decreased with increasing concentrations of GABA. The EC_{35} were plotted in the isobologram (Fig. 3). The total drug load was calculated as given in Materials and methods. If the total drug load is below unity, then there is synergy. The Hill coefficients were not different from unity. The number of experiments in triplicate is indicated by n.

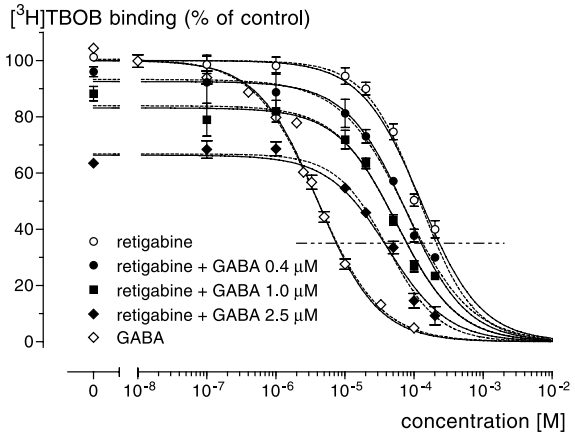

Fig. 2. The displacement curves of GABA and retigabine in the presence of several concentrations of GABA on the binding of [^{3}H]TBOB (8 nM) (data points: mean ± S.E.M., $n=4-6$ in triplicate). Dotted lines indicate the sigmoid-E_{max} model (Eq. (1)) fitted to the data. Parameter estimates are given in Table 1. Solid lines indicate the allosteric three-ligand molecular model fitted to the data. Parameters estimated for the K_d values and γ, quantifying the coupling between GABA and retigabine, are given in Table 2. The stripped-dotted line indicates the 35% binding level.

3.2. Isobolic analysis

An isobologram was constructed for 35% binding of [^{3}H]TBOB (Fig. 3). The experimentally determined data points of combinations of GABA and retigabine were below the isobole, i.e. below the theoretical additive values. ED_{35} estimates and total drug loads are given in Table 1. For the two highest GABA concentrations, there was no overlap between the 95% confidence intervals of the theoretical additive concentrations and the actually measured concentrations, so the isobolic analysis showed that retigabine interacts synergistically with GABA in displacing [^{3}H]TBOB at 35% binding.

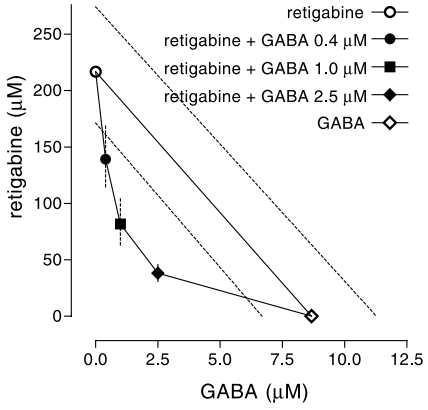

Fig. 3. Isobologram of combinations of retigabine with GABA for the 35% binding. Abscissa: concentration of GABA; ordinate: concentration of retigabine. The open symbols plotted on the axis represent the EC_{35} values of the pure compounds, defining the isobole. Closed symbols represent observed concentrations of retigabine in the presence of GABA. Dotted lines indicate the 95% confidence intervals. Since the experimental points lie below the isobole, this figure demonstrates synergy between retigabine and GABA in displacing [^{3}H]TBOB.

Table 2
Parameter estimates of the allosteric three-ligand molecular model fitted to the experimental data

Drug	K_d (μM) (95% CI)	γ (95% CI)
GABA (K_5)	2.5 (2.0, 2.9)	
Retigabine (K_9)	64 (55, 74)	
Retigabine + GABA 0.4		0.20, (0.14, 0.27)
Retigabine + GABA 1		0.24, (0.16, 0.32)
Retigabine + GABA 2.5		0.28, (0.20, 0.36)

The simplified allosteric three-ligand molecular model (Eq. (3)) was fitted to the experimental data, yielding K_d values for GABA and retigabine and the interaction term γ. Best-fit values and 95% confidence intervals of fit are given. The K_d value of TBOB (K_1) was assumed to be 8 nM (Van Rijn et al., 1990). The parameter γ quantifies the coupling between GABA and retigabine. The three γ's were not significantly different (see 95% CI). GABA enhances the affinity of the retigabine and vice versa with a factor $1/\gamma$.

3.3. Molecular modelling

In order to achieve complete displacement of [^{3}H]TBOB binding by retigabine, by GABA and by the combination of the two drugs, the factors β and δ and ε must be large ($\approx 1\times10^6$). Thus, the equation of the molecular model can be simplified to:

$$\frac{[\mathrm{Bound_{tot}}]}{[\mathrm{R_{tot}}]} = \frac{\frac{[\mathrm{T}]}{K_1}}{2+\frac{[\mathrm{A}]}{K_9}+\frac{[\mathrm{G}]}{K_5}+\frac{[\mathrm{A}][\mathrm{G}]}{\gamma K_5 K_9}} \quad (3)$$

This leaves only the following parameters to be estimated: K_1 (K_{TBOB}), K_5 (K_{GABA}), K_9 ($K_{retigabine}$) and γ. K_1 was assumed to be 8 nM (Van Rijn et al., 1990).

The right-hand side of Eq. (3) was fitted to the experimental data. Fig. 2 shows the fitted curves. The parameter estimates are given in Table 2. The mean allosteric interaction term γ was 0.25 [CI (0.17, 0.32)], so GABA and retigabine enhance each other's affinity for binding to the $GABA_A$ receptor complex by 4.0-fold [CI (5.9, 3.1)].

4. Discussion

In this study, we investigated whether retigabine interacts directly with the $GABA_A$ receptor complex and, if so, whether it interferes with the binding of GABA. The effects of retigabine and GABA were measured indirectly using [^{3}H]TBOB as a tracer ligand. Retigabine indeed displaced [^{3}H]TBOB, and GABA shifted the displacement curves of retigabine to the left. Isobolic analysis of these results showed the interaction between retigabine and GABA to be synergistic. The isobole method, however, describes interactions only in empirical terms and is not appropriate to describe data in mechanistic terms; physiological molecular models are needed for this (Berenbaum, 1989; Greco et al., 1995).

We showed that a simple molecular model could describe the experimental data adequately. This three-ligand molecular model is described by Eq. (2). Earlier we reported a more extensive three-ligand model (Van Rijn et al., 1999), but because the Hill coefficient of retigabine is not different from unity, this simple model can describe the present experimental data. According to this model, retigabine and GABA enhance each other's affinity with a factor of 4.

The $GABA_A$ receptor complex comprises binding sites for a variety of compounds (Mehta and Ticku, 1999). We do not know whether retigabine interacts with one of these known receptor sites or whether it interacts with a new, yet unidentified site. It has been shown before that retigabine does not interact with the benzodiazepine site since the benzodiazepine antagonist flumazenil does not block the effect of retigabine on GABA-induced chloride currents (Rundfeldt and Netzer, 2000). Therefore the binding site for retigabine on the $GABA_A$ complex remains to be characterized.

We showed that retigabine enhances GABA-ergic activity in vivo. However, it was suggested before that the contribution to the clinical effect of retigabine of a GABA-ergic enhancement will be small since the potency of retigabine to enhance Cl^- currents is less than the potency for enhancing K^+ currents (Rundfeldt and Netzer, 2000). However, our findings suggest that the contribution of the GABA enhancement to the clinical effect might be far from negligible. The plasma concentration of retigabine in rats as well as in humans is about 3–10 μM (Otto et al., 2002). At these concentrations, in the presence of GABA, the dose–effect curves for displacing [^{3}H]TBOB are at the beginning of the steep parts. Otto et al. (2002) showed that retigabine is most effective in increasing peak amplitudes and decay time of inhibitory postsynaptic currents under conditions in which the synapse is not saturated by GABA. Our results support this observation: a left shift of a dose–effect curve is most effective if the steep part of the curve shifts. Moreover, it will depend on the network properties and relative contribution to the inhibition of convulsions whether the chloride currents, the potassium currents or the interaction between them will mainly contribute to the inhibition of seizure activity. Otto et al. (2002) showed that both mechanisms add to a decrease in excitability in neural circuits. They hypothesise a synergistic interaction between the two mechanisms but it needs further analysis to show whether the interaction is synergistic in isobolic terms or merely additive.

In summary, we found that retigabine does indeed interact with a site on the $GABA_A$ receptor complex, and that this site is positively allosterically coupled with the GABA site. The potency of retigabine to enhance the effects of GABA was found to be less than its reported potency to enhance potassium currents. However, the relative contribution of these retigabine effects to the antiepileptic effect in vivo will depend on their weight in the complex neural network participating in seizure activity.

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Appendix A

The following expressions for the dissociation constants (K_d's) of the drug–receptor complexes hold for the model depicted in Fig. 1:

$$K_1 = \frac{[R][T]}{[RT]} \quad K_2 = \frac{[RA][T]}{[RAT]} \quad K_3 = \frac{[RG][T]}{[RTG]} \quad K_4 = \frac{[RAG][T]}{[RATG]}$$

$$K_5 = \frac{[R][G]}{[RG]} \quad K_6 = \frac{[RA][G]}{[RAG]} \quad K_7 = \frac{[RT][G]}{[RTG]} \quad K_8 = \frac{[RAT][G]}{[RATG]}$$

$$K_9 = \frac{[R][A]}{[RA]} \quad K_{10} = \frac{[RG][A]}{[RAG]} \quad K_{11} = \frac{[RT][A]}{[RAT]} \quad K_{12} = \frac{[RTG][A]}{[RATG]}$$

$$K_1K_7 = K_5K_3$$

$$K_2K_8 = K_6K_4$$

$$K_1K_{11} = K_9K_2$$

$$K_3K_{12} = K_{10}K_4$$

$$K_5K_{10} = K_9K_6$$

$$K_7K_{12} = K_{11}K_8$$

To describe the allosteric interactions the following factors are introduced:

β	describes the change in K_d of TBOB by the binding of retigabine: $K_2 = \beta K_1$
γ	describes the change in K_d of GABA by the binding of retigabine: $K_6 = \gamma K_5$
δ	describes the change in K_d of TBOB by the binding of GABA: $K_3 = \delta K_1$
ε	describes the change in K_d of TBOB by the binding of both GABA and retigabine: $K_4 = \varepsilon K_3 = \varepsilon\delta K_1$

The concentration of [^{3}H]TBOB bound is:

$$[\mathrm{Bound_{tot}}] = [RT] + [RAT] + [RTG] + [RATG]$$

The total receptor concentration is the sum of free receptors and occupied receptors:

$$[\mathrm{R_{tot}}] = [R]\left\{1 + \frac{[A]}{K_{13}} + \frac{[G]}{K_5} + \frac{[A][G]}{\gamma K_5 K_9} + \frac{[T]}{K_1} \times \left(1 + \frac{[A]}{\beta K_9} + \frac{[G]}{\delta K_5} + \frac{[A][G]}{\gamma\delta\varepsilon K_9 K_5}\right)\right\}$$

The displacement curves are described in terms of fractional occupancy:

$$\frac{[\mathrm{Bound_{tot}}]}{[\mathrm{R_{tot}}]}$$

Rearrangement of the above equations yields:

$$[\mathrm{Bound_{tot}}] = \frac{[\mathrm{R}][\mathrm{T}]}{K_1}\left(1 + \frac{[\mathrm{A}]}{\beta K_9} + \frac{[\mathrm{G}]}{\delta K_5} + \frac{[\mathrm{A}][\mathrm{G}]}{\gamma\delta\varepsilon K_5 K_9}\right)$$

$$[\mathrm{R_{tot}}] = [\mathrm{R}]\left\{1 + \frac{[\mathrm{A}]}{K_9} + \frac{[\mathrm{G}]}{K_5} + \frac{[\mathrm{A}][\mathrm{G}]}{\gamma K_5 K_9} + \frac{[\mathrm{T}]}{K_1} \times \left(1 + \frac{[\mathrm{A}]}{\beta K_9} + \frac{[\mathrm{G}]}{\delta K_5} + \frac{[\mathrm{A}][\mathrm{G}]}{\gamma\delta\varepsilon K_5 K_9}\right)\right\}$$

Thus the overall expression describing the molecular model labelled with [^{3}H]TBOB is given in Eq. (2).

The equation was fitted to the data using nonlinear regression analysis. [T] is the concentration of [^{3}H]TBOB, [A] is the concentration of retigabine and [G] is the concentration of GABA. The parameters K_1, K_5, K_9, β, γ, δ and ε were to be estimated.

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