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Stoichiometry of a Recombinant $GABA_A$ Receptor

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GABA is the main inhibitory neurotransmitter in the mammalian brain. The postsynaptic $GABA_A$ receptor/pore complex is presumed to be a pentamer typically composed of a combination of α, β, and γ subunits, although the stoichiometry remains controversial. We probed the stoichiometry of the $GABA_A$ receptor by site-directed mutagenesis of a conserved leucine (to serine) in the putative second membrane-spanning domain of the rat $\alpha1$(αL263S), $\beta2$(αL259S), and $\gamma2$(αL274S) subunit isoforms. Coexpression of wild-type and mutant subunits of each class (e.g., α and αL263S), along with their wild-type counterparts (e.g., β and γ), in *Xenopus laevis* oocytes resulted in mixed populations of receptors with distinct GABA sensitivities. This is consistent with the interpretation that the leucine mutation increased the GABA sensitivity in proportion to the number of incorporated mutant subunits. The apparent number of incorporated subunits for each class (α, β, and γ) could then be determined from the number of components comprising the compound GABA dose–response relationships. Using this approach, we conclude that the recombinant $\alpha1\beta2\gamma2$ $GABA_A$ receptor is a pentamer composed of two α subunits, two β subunits, and one γ subunit.

Key words: GABA; stoichiometry; receptor; ion channel; mutagenesis

The release of GABA from presynaptic nerve terminals in the CNS inhibits the postsynaptic neuron by gating a chloride-selective ion pore that is an integral component of the receptor complex. Four different classes of $GABA_A$ receptor subunits have thus far been identified in the mammalian brain: α, β, γ, and δ (Schofield et al., 1987; Khrestchatisky et al., 1989; Lolait et al., 1989; Shivers et al., 1989; Ymer et al., 1989; Harvey et al., 1993). In addition, multiple isoforms have been isolated for each of the α, β, and γ subunit classes. Exogenous expression of various combinations of subunits suggests that $GABA_A$ receptors must contain α, β, and γ subunits to reconstitute the major features of native $GABA_A$ receptors (Levitan et al., 1988; Pritchett et al., 1989; Malherbe et al., 1990; Sigel et al., 1990; Verdoorn et al., 1990). Although structure–function studies of recombinant receptors have elucidated subunit domains and residues critical for activation and modulation (Pritchett and Seeburg, 1991; Sigel et al., 1992; Wieland et al., 1992; Amin and Weiss, 1993), the stoichiometry of the $GABA_A$ receptor has been elusive.

Mutation of a conserved leucine in the putative second membrane-spanning domain of homomeric neuronal nicotinic acetylcholine (nACh) and serotonin type 3 ($5\text{-}HT_3$) receptors induced an increase in agonist sensitivity (Revah et al., 1991; Yakel et al., 1993). (nACh and $5\text{-}HT_3$ receptors are members of the same ligand-operated ion channel superfamily as the $GABA_A$ receptor.) More recent studies of heteromeric nACh receptors determined that each subunit of the receptor complex carrying this leucine mutation contributed an ≈10-fold increase in ACh sensitivity (Filatov and White, 1995; Labarca et al., 1995).

We have used this leucine mutation to infer the number of each subunit type that comprises recombinant $\alpha1\beta2\gamma2$ GABA receptors expressed in *Xenopus* oocytes. Mutation of this leucine to serine in either the rat $\alpha1$, $\beta2$, or $\gamma2$ GABA subunit isoforms increased the sensitivity to GABA. Coexpression of wild-type and mutant subunits of each class (e.g., α and α-mutant), along with their wild-type counterparts (e.g., β and γ), resulted in mixed populations of receptors with distinct GABA sensitivities. We were then able to infer the number of each subunit type from the number of components comprising these compound GABA dose–response relationships. Using this approach, we conclude that the $\alpha1\beta2\gamma2$ GABA receptor is a pentamer composed of two α subunits, two β subunits, and one γ subunit.

MATERIALS AND METHODS

Site-directed mutagenesis and in vitro *transcription.* The cDNAs were isolated by the PCR (Saiki et al., 1988) as described previously (Amin et al., 1994). The cDNAs were cloned into the pALTER vector for oligonucleotide-mediated site-directed mutagenesis using Altered Sites (Promega, Madison WI). The oligonucleotides (complementary to the sense strand) used to make the leucine to serine substitutions were $\alpha1$(L263S): 5′-CAA GGT TGT CAT GGT **ACT** AAC GGT CGT CAC TCC-3′; $\beta2$(L259S): 5′-GAT TGT GGT CAT CGT **ACT** GAC AGT TGT AAT TCC-3′; and $\gamma2$(L274S): 5′-GAG AGT GGT CAT CGT **ACT** GAC AGT CGT GAT TCC-3′.

The mismatched base pairs are indicated in bold. Successful mutagenesis was confirmed by sequencing (Sanger et al., 1977). cDNAs were linearized with *Ssp*I, which leaves a several hundred base pair tail that may increase cRNA stability in the oocyte. cRNA was transcribed from the linearized cDNAs by standard *in vitro* transcription procedures. Methods used to match the cRNA concentrations of the three subunits have been described previously (Amin and Weiss, 1996).

Oocyte injection. Xenopus laevis (Xenopus I, Ann Arbor, MI) were anesthetized by hypothermia, and ovarian lobes were surgically removed from the frog and placed in a solution that consisted of (in mM): 82.5 NaCl, 2.5 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, and 10 Na_2HPO_4, 50 U/ml penicillin, and 50 μg/ml streptomycin, pH 7.5. Oocytes were dispersed in this same solution minus $CaCl_2$ plus 0.3% Collagenase A (Boehringer Mannheim, Indianapolis, IN). After isolation, the oocytes were rinsed thoroughly. Stage VI oocytes were separated and maintained overnight at 18°C.

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Micropipettes for injecting cRNA were fabricated on a Sutter P87 horizontal puller, and the tips were cut off with microscissors. The wild-type and mutant cRNAs were then mixed at the desired ratios and diluted with diethyl pyrocarbonate-treated water. Except for the experiments presented in Figure 5, the α:β:γ cRNA injection ratio was always 1:1:1. The cRNA mixture was then drawn up into the micropipette and injected with the Nanoject injector (Drummond Scientific, Broomall, PA).

Electrophysiological recording. One to three days after injection, oocytes were placed on a 300 μm nylon mesh suspended in a small volume chamber (<100 μl). The chamber and perfusion system, which allows up to 18 different solutions to be introduced to an individual oocyte, has been described previously (Amin et al., 1994). The oocyte was perfused continuously with a solution that consisted of (in mM): 92.5 NaCl, 2.5 KCl, 10 HEPES, 2 $CaCl_2$, and 1 $MgCl_2$, pH 7.5, and briefly switched to the test solution containing GABA.

Recording microelectrodes were fabricated with a P87 Sutter horizontal puller and filled with 3 M KCl. They had resistances of 1–3 MΩ. Standard two-electrode voltage-clamp techniques were used to record currents in response to the application of GABA. In all cases, the membrane potential was clamped to −70 mV. Data were played out on a chart recorder during the experiment and recorded on tape for off-line analysis.

Peak current amplitudes were measured directly from the chart record or from the computer screen. To quantify the agonist sensitivity, each dose–response relationship was fitted with the following equation using a nonlinear least-squares method:

$$I = \sum_{j=1}^{x} \frac{I_{\max_j}}{1+(EC_{50_j}/[A])^{n_j}},$$

where x is the number of fitted components and can vary from 1 to 3, I is the peak current at a given concentration of GABA (A), I_{max} is the maximum current, EC_{50} is the concentration of GABA yielding a current half the maximum, and n is the Hill coefficient.

RESULTS

Mutation of the conserved leucine in α1, β2, or γ2 increases the GABA sensitivity

The isoforms α1, β2, and γ2 are widely distributed in the brain (Benke et al., 1991; Wisden et al., 1992; Ruano et al., 1994), so we selected the α1β2γ2 combination for this particular study. Given the remarkable sequence homology between isoforms within a class, the conclusions we reach will likely apply to recombinant GABA receptors composed of other α, β, and γ isoforms. In this manuscript, we designate the rat α1, β2, and γ2 subunit isoforms as simply α, β, and γ.

A conserved leucine in the putative second membrane-spanning domain (TM2) was mutated to serine in the α (αL263S), β (βL259S), and γ (γL274S) subunits. (Henceforth, the subunits with the leucine mutation will be designated α_m, β_m, and γ_m). cRNA was *in vitro* transcribed for both mutant and wild-type α, β, and γ subunits, mixed in the combinations αβγ, $\alpha_m\beta\gamma$, $\alpha\beta_m\gamma$, and $\alpha\beta\gamma_m$, and injected into *Xenopus laevis* oocytes. Figure 1*A* shows examples of GABA-activated currents from each of the four combinations of subunits. Note that much lower concentrations of GABA were required to activate the GABA receptors containing either an α_m, β_m, or γ_m subunit. Figure 1*B* shows average GABA dose–response relationships for all four subunit combinations and illustrates the leftward shifts in the GABA dose– response relationships induced by the leucine to serine substitutions. The continuous lines are the best fits of the Hill equation to the data. The EC_{50} values (concentration of GABA required for half-maximal activation) determined from the fits were (mean ± SD) αβγ, 45.8 ± 3.6 μM; $\alpha_m\beta\gamma$, 0.30 ± 0.13 μM; $\alpha\beta_m\gamma$, 0.035 ± 0.004 μM; and $\alpha\beta\gamma_m$, 0.99 ± 0.23 μM.

Studies of heteromeric nACh receptors, in which the stoichiometry has been established (Reynolds and Karlin, 1978; Lindstrom et al., 1979; Raftery et al., 1980), concluded that each subunit of the receptor complex carrying this leucine mutation contributed an ≈10-fold increase in ACh sensitivity (Filatov and White, 1995; Labarca et al., 1995). For example, if two subunits in the nACh receptor complex are mutated, an ≈100-fold increase in ACh sensitivity was observed. In this study, we are attempting to work in the other direction; that is, infer the number of each subunit type comprising the $GABA_A$ receptor from the shift in GABA sensitivity induced by the mutations. The data presented in Figure 1 demonstrate a 153-, 1308-, and 46-fold decrease in the EC_{50} with α_m, β_m, and γ_m, respectively. Because the effects of the mutations may not be equivalent for α, β, and γ subunits, we could not ascertain directly the stoichiometry from these observed shifts in GABA sensitivity. Similarly, the shift in ACh sensitivity for the nACh receptor with one mutant subunit varied from 13-fold to 49-fold depending on which particular subunit (α, β, γ, or δ) carried the mutation (Labarca et al., 1995). We now describe a strategy to ascertain the stoichiometry that is not compromised by differences in the magnitude of the shifts induced by α_m, β_m, and γ_m.

Coexpression of wild-type and mutant subunits

Predictions

Assume there is only one α subunit in the $GABA_A$ receptor complex. Coexpression of both α and α_m subunits along with wild-type β and γ subunits would result in GABA dose–response relationships having two components (Fig. 2*A*): one component from activation of the $\alpha_m\beta\gamma$ receptors and one component from activation of the αβγ receptors. Furthermore, on the basis of the data presented in Figure 1, the GABA EC_{50} values of these two components would be ≈0.30 and ≈46 μM, respectively. Alternatively, if the GABA receptor complex contained two α subunits, the GABA dose–response relationships resulting from coexpression of α_m, α, β, and γ subunits would have three components (Fig. 2*B*): one component from receptors in which both α subunits are wild type (EC_{50} ≈46 μM), one component from receptors in which both α subunits are mutant (EC_{50} ≈0.30 μM), and one component with an intermediate GABA sensitivity from receptors with one wild-type and one mutant α subunit. If mutation of the two α subunits has an equivalent effect, the shift in sensitivity contributed by each α_m subunit would be the square root of the shift in EC_{50} observed when both α subunits are mutant (153-fold; Fig. 1*B*). Thus, each α_m subunit would contribute a 12.4-fold increase in GABA sensitivity predicting an intermediate component with an EC_{50} of 3.7 μM. (Similar logic could be applied to arrive at the four components that would result from $\alpha\alpha_m\beta\gamma$ coexpression, assuming three α subunits in the receptor complex.)

$\alpha\alpha_m\beta\gamma$ coexpression

Figure 3*A* shows the results from experiments in which α_m, α, β, and γ subunits were all coexpressed in the same oocyte. The resulting dose–response relationship was well described by the sum of three Hill equations (continuous line). The EC_{50} of the first component, 0.26 ± 0.05 μM, corresponds to that determined with $\alpha_m\beta\gamma$ coexpression (0.30 ± 0.13 μM), and the EC_{50} of the third component, 36.3 ± 8.1 μM, corresponds to that of the αβγ receptor (45.8 ± 3.6 μM). The presence of an intermediate component (EC_{50} = 2.2 ± 0.1 μM) indicates that the GABA receptor must contain more than one α subunit (Fig. 2*B*). The EC_{50} of this intermediate component is in excellent agreement with the predicted intermediate EC_{50} of 3.7 μM (Fig. 2*B*). Our interpretation

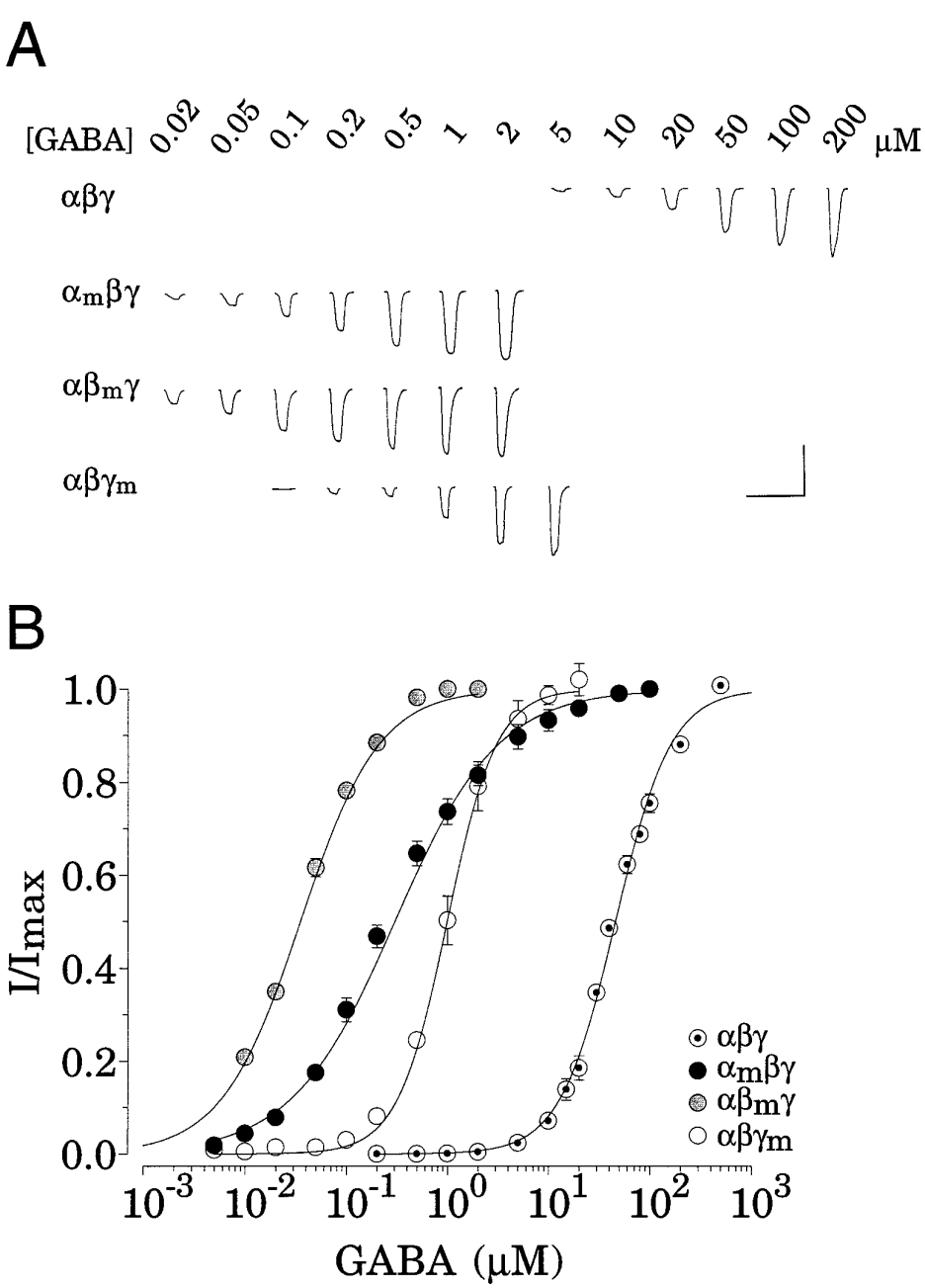

Figure 1. Mutation of the conserved leucine in TM2 of the α1(L263S), β2(L259S), and γ2(L274S) subunit increases the GABA sensitivity of the $GABA_A$ receptor. *A*, GABA-activated currents from oocytes expressing $\alpha\beta\gamma$, $\alpha_m\beta\gamma$, $\alpha\beta_m\gamma$, and $\alpha\beta\gamma_m$ subunit combinations. The *subscript "m"* indicates that the conserved leucine in TM2 was mutated to serine. GABA was bath-applied at the indicated concentrations. Note the increase in GABA sensitivity induced by the mutation in each subunit. Calibration: 100 sec; 350, 65, 35, and 500 nA for the four rows of traces, respectively. *B*, Average GABA dose–response relationships for each of the four combination of subunits (mean ± SEM). The *continuous lines* are the best fit of the Hill equation to the data points. The EC_{50} values and Hill coefficients (mean ± SD) for the fits are $\alpha\beta\gamma$: 45.8 ± 3.6 μM, 1.59 ± 0.09 (n = 5); $\alpha_m\beta\gamma$: 0.30 ± 0.050 μM, 0.85 ± 0.10 (n = 9); $\alpha\beta_m\gamma$: 0.035 ± 0.004 μM, 1.12 ± 0.04 (n = 3); and $\alpha\beta\gamma_m$: 0.99 ± 0.23 μM, 1.78 ± 0.36 (n = 4).

of this intermediate component is that it represents the activation of receptors containing both an α and an α_m subunit. This interpretation is supported by the experiment shown in the inset of Figure 3*A*. Oocytes were injected with $\alpha\beta\gamma$ cRNA; 5 d later, the same oocytes were injected with $\alpha_m\beta\gamma$ cRNA. Previous studies demonstrate that the cRNA for glycine subunits (Kuhse et al., 1993) or GABA subunits (Amin and Weiss, 1996) injected into *Xenopus* oocytes is degraded within 2–3 d. Therefore, after the second injection, the oocytes should contain only $\alpha\beta\gamma$ and $\alpha_m\beta\gamma$ receptors, and no intermediate component (attributable to $\alpha\alpha_m\beta\gamma$ receptors) should be observed. This was indeed the case, because the dose–response relationship in these delayed-injection experiments was described by the sum of two Hill equations (continuous line) with EC_{50} values of (mean ± SD) 0.42 ± 0.17 μM and 30.0 ± 7.5 μM (n = 3).

$\alpha\beta\beta_m\gamma$ coexpression

Figure 3*B* presents the average GABA dose–response relationship from oocytes coexpressing β and β_m subunits along with wild-type α and γ subunits. The continuous line in Figure 3*B* is the best fit of the sum of three Hill equations. The EC_{50} of the first component, 0.025 ± 0.01 μM, corresponds to that determined with $\alpha\beta_m\gamma$ coexpression (0.035 ± 0.004 μM), and the EC_{50} of the third component, 39.2 ± 7.9 μM, corresponds to that of the $\alpha\beta\gamma$ receptor (45.8 ± 3.6 μM). Again, the presence of an intermediate component (EC_{50} = 0.94 ± 0.07 μM) indicates that the GABA receptor contains more than one β subunit (Fig. 2*B*). Assuming two β subunits and the shift observed in Figure 1*B* for $\alpha\beta_m\gamma$ coexpression (1308-fold), each β_m subunit would contribute a 36.2-fold increase in GABA sensitivity, predicting an intermediate EC_{50} of 1.27 μM. This is

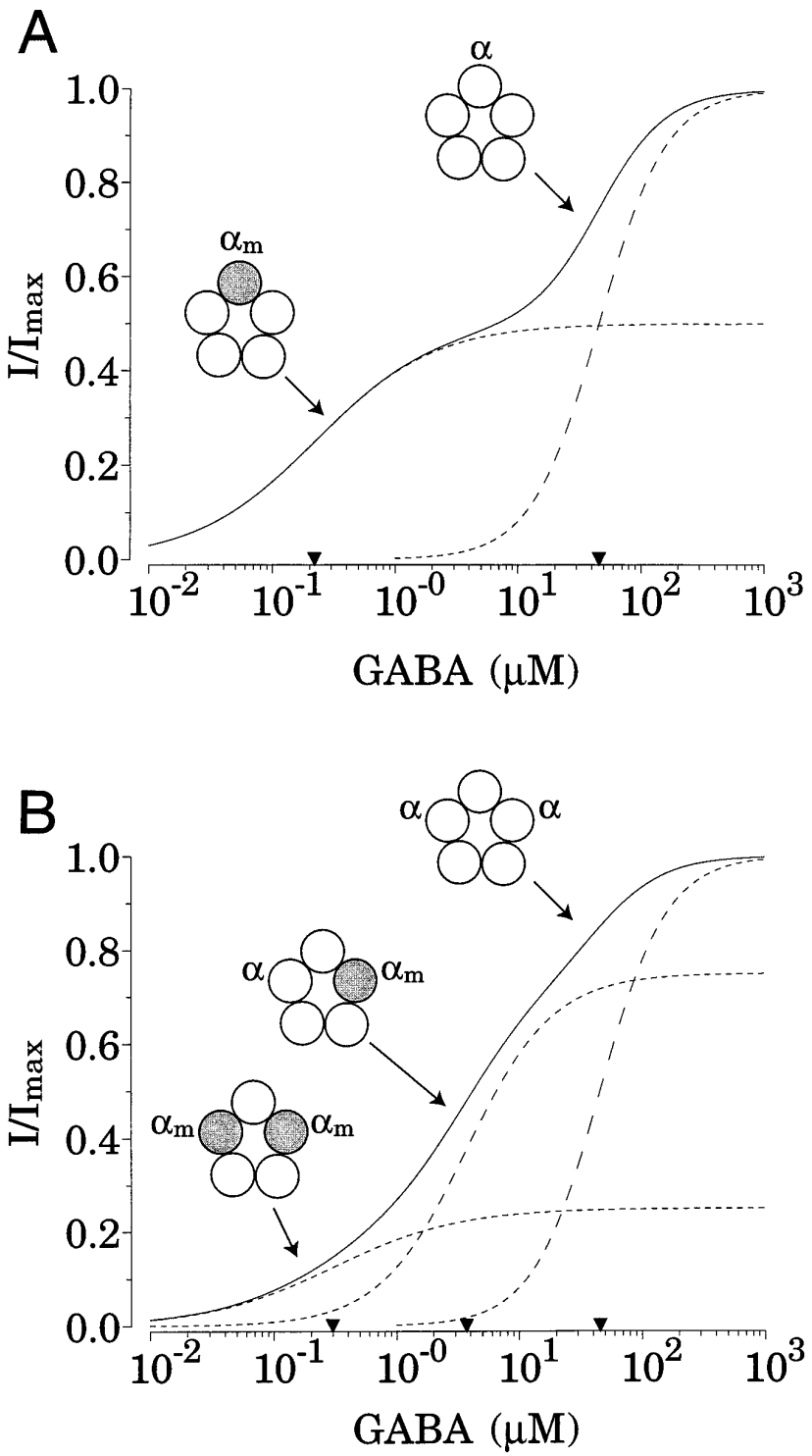

Figure 2. The number of components comprising the GABA dose–response relationship with $\alpha\alpha_m\beta\gamma$ coexpression depends on the number of α subunits in the GABA receptor complex. *A*, With one α subunit in the GABA receptor complex, the GABA dose–response relationship from oocytes coexpressing α, α_m, β, and γ subunits would be composed of two components (*continuous line*): one component from activation of $\alpha\beta\gamma$ receptors and one component from activation of $\alpha_m\beta\gamma$ receptors (*dashed lines*, scaled to facilitate comparison with the *continuous line*). The EC_{50} values of these two components would be ≈0.30 and ≈45.8 μM (indicated on the abscissa) as demonstrated by the data in Figure 1. The mutant α subunit is shown *shaded*. *B*, With two α subunits in the GABA receptor complex, the GABA dose–response relationship from oocytes coexpressing α, α_m, β, and γ subunits would be composed of three components (*continuous line*): one component from activation of $\alpha\beta\gamma$ receptors, one component from activation of $\alpha_m\beta\gamma$ receptors, and an intermediate component from activation of receptors containing both an α and an α_m subunit (*dashed lines*). The EC_{50} values of the first and third components would be ≈0.30 and ≈45.8 μM, and the predicted EC_{50} of the intermediate component, assuming the two α_m subunits contribute equally to the shift, would be 3.7 μM. A similar logic could be applied to $\alpha\beta\beta_m\gamma$ and $\alpha\beta\gamma\gamma_m$ coexpression to determine the number of β and γ subunits, respectively.

in excellent agreement with the observed intermediate EC_{50} of 0.94 ± 0.07 μM.

$\alpha\beta\gamma\gamma_m$ coexpression

The data presented thus far (Fig. 3*A,B*) suggest the presence of two α subunits and two β subunits. If, by analogy with other members of this receptor-operated super-family, we assume that the $GABA_A$ receptor is a pentameric complex (Langosch et al., 1988; Anand et al., 1991; Cooper et al., 1991; Unwin, 1993; Nayeem et al., 1994; Macdonald and Olsen, 1994), we can assume there is only one γ subunit. This inferred presence of one γ subunit is confirmed by the $\alpha\beta\gamma\gamma_m$ coexpression studies presented in Figure 3*C*. In contrast to that of $\alpha\alpha_m\beta\gamma$ and $\alpha\beta\beta_m\gamma$ coexpression, the $\alpha\beta\gamma\gamma_m$ dose–response relationships were described by the sum of only two Hill equations (continuous line). Furthermore, the EC_{50} of the first component, 1.09 ± 0.12 μM, corresponds to that determined with $\alpha\beta\gamma_m$ coexpression (0.99 ± 0.23), and the EC_{50} of the second component, 40.9 ± 4.8 μM, corresponds to that determined for $\alpha\beta\gamma$ receptors (45.8 ± 3.6 μM).

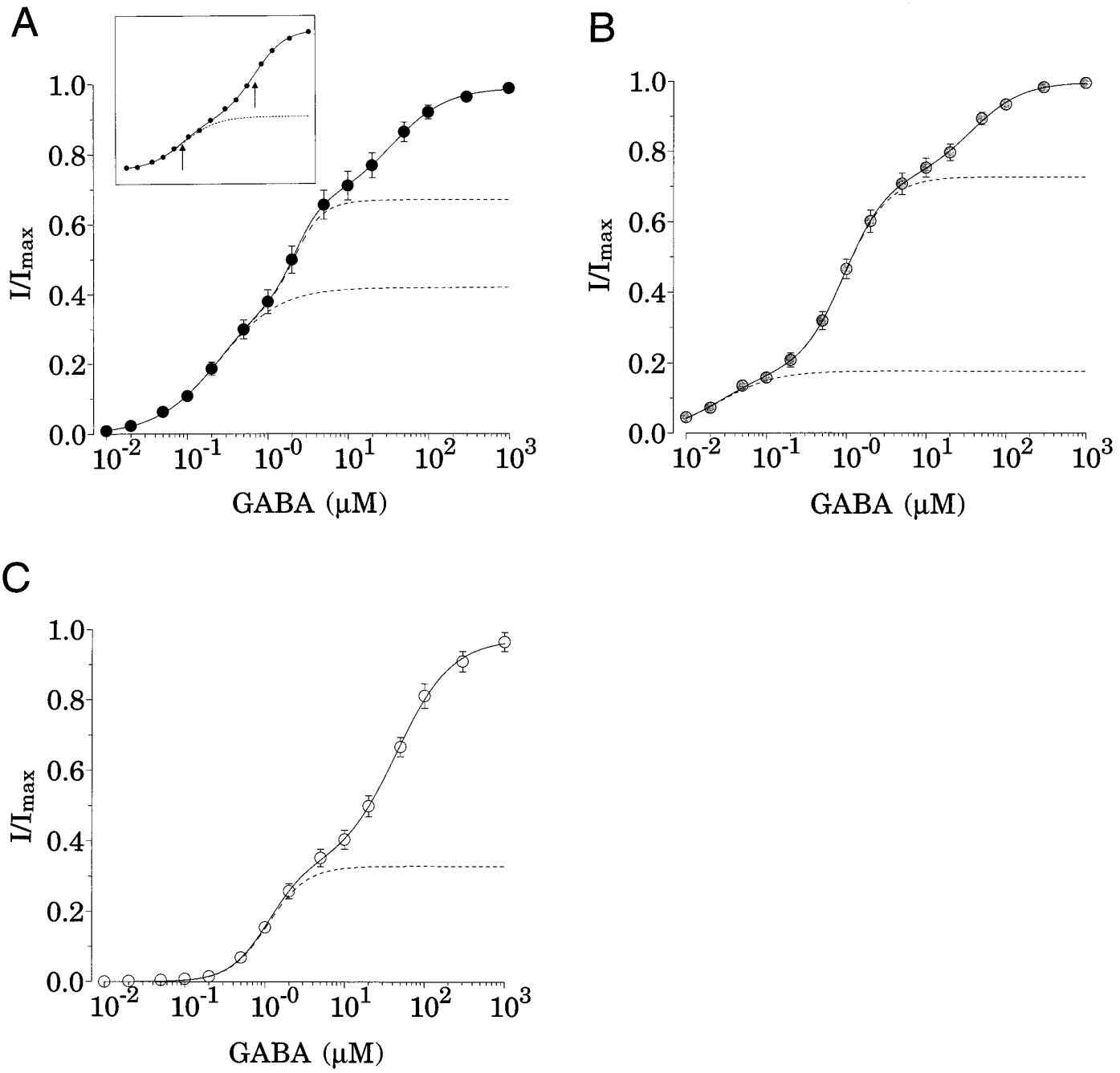

Figure 3. GABA dose–response relationships from coexpression of $\alpha\alpha_m\beta\gamma$, $\alpha\beta\beta_m\gamma$, and $\alpha\beta\gamma\gamma_m$ subunits. *A*, cRNA encoding for α, α_m, β, and γ subunits was coinjected into oocytes. The dose–response relationship for GABA (mean ± SEM, $n = 4$) was well described by the sum of three Hill equations (*continuous line*), suggesting, in terms of GABA sensitivity, three receptor subtypes. The EC_{50} values of the three components were 0.26 ± 0.05, 2.2 ± 0.1, and 36.3 ± 8.1 μM (α-to-α_m cRNA injection ratio = 1:3). The *dashed lines* representing the first component and the combination of the first and second component are shown to delineate the individual components. The three components suggest that there are two α subunits in the $GABA_A$ receptor complex. The *inset* is a GABA dose–response relationship from an oocyte expressing primarily $\alpha\beta\gamma$ and $\alpha_m\beta\gamma$ receptors. This was achieved by injecting $\alpha\beta\gamma$ cRNA, waiting 5 d, and then injecting $\alpha_m\beta\gamma$ cRNA. This ensures that α and α_m cRNAs do not coexist to any appreciable extent in the oocyte. In this case there was no intermediate component, and the GABA dose–response relationship was described by the sum of two Hill equations with EC_{50} values of 0.34 and 33.4 μM (indicated by *arrows*). The EC_{50} values from three such experiments (mean ± SD) were 0.42 ± 0.17 and 30.0 ± 7.5 μM for the first and second components, respectively. *B*, cRNA encoding for α, β, β_m, and γ subunits was coinjected into oocytes. The dose–response relationship for GABA (mean ± SEM, $n = 3$) was also well described by the sum of three Hill equations (*continuous line*). The EC_{50} values of the three components were 0.025 ± 0.01, 0.94 ± 0.07, and 39.2 ± 7.9 μM (β-to-β_m cRNA injection ratio = 1:1). The three components suggest that there are two β subunits in the $GABA_A$ receptor complex. *C*, cRNA encoding for α, β, γ, and γ_m subunits was coinjected into oocytes. The dose–response relationship for GABA (mean ± SEM, $n = 3$) was described by the sum of two Hill equations (*continuous line*) with EC_{50} values of 1.09 ± 0.12 and 40.9 ± 4.8 μM (γ-to-γ_m cRNA injection ratio = 1:1). The *dashed line* represents the first component. Two components suggest that there is one γ subunit in the $GABA_A$ receptor complex.

Table 1. EC_{50} values and fractions of the components for the different wild-type/mutant cRNA injection ratios

cRNA injection ratio	First component		Second component		Third component		
	EC_{50} (μM)	Fraction	EC_{50} (μM)	Fraction	EC_{50} (μM)	Fraction	*n*
$\alpha\alpha_m(1:3)\beta\gamma$	0.26 ± 0.05	0.43 ± 0.08	2.2 ± 0.1	0.24 ± 0.13	36.3 ± 8.1	0.33 ± 0.15	4
$\alpha\alpha_m(1:1)\beta\gamma$	0.41 ± 0.08	0.17 ± 0.05	2.0 ± 0.2	0.10 ± 0.02	42.4 ± 10.4	0.72 ± 0.07	3
$\alpha\alpha_m(3:1)\beta\gamma$	0.35 ± 0.17	0.05 ± 0.04	2.0 ± 0.1	0.09 ± 0.02	40.1 ± 5.7	0.86 ± 0.01	2
$\alpha\beta\beta_m(1:1)\gamma$	0.025 ± 0.01	0.17 ± 0.03	0.94 ± 0.07	0.6 ± 0.04	39.2 ± 7.9	0.27 ± 0.07	4
$\alpha\beta\beta_m(3:1)\gamma$	0.038 ± 0.01	0.03 ± 0.01	0.92 ± 0.05	0.3 ± 0.02	43.4 ± 0.9	0.63 ± 0.02	3
$\alpha\beta\gamma\gamma_m(1:3)$	1.16 ± 0.14	0.58 ± 0.12	47.9 ± 19.8	0.42 ± 0.12			3
$\alpha\beta\gamma\gamma_m(1:1)$	1.09 ± 0.12	0.28 ± 0.12	40.9 ± 4.8	0.72 ± 0.12			3

Values are mean ± SD. In all cases, total α:β:γ cRNA ratios were 1:1:1.

Therefore, the two components in Figure 3*C* represent the activation of receptors containing either a γ or γ_m subunit, and the GABA receptor complex contains only one γ subunit.

Varying the ratio of wild-type and mutant subunits

We next considered the possibility that there are multiple indistinguishable intermediate components in the dose–response relationships of Figure 3. To address this issue, the ratio of wild-type and mutant cRNA (e.g., α and α_m) coinjected with their wild-type counterparts (e.g., β and γ) was varied. For example, if the intermediate component of the GABA dose–response relationship in Figure 3*A* consists of more than one component, then varying the α-to-α_m cRNA ratio should vary the relative fractions of the different receptor combinations underlying the intermediate components and shift the apparent EC_{50} of the intermediate component. A similar argument would apply for changes in the β-to-β_m and γ-to-γ_m cRNA ratio.

At all cRNA injection ratios tested, the GABA dose–response relationships consisted of three components for $\alpha\alpha_m\beta\gamma$ and $\alpha\beta\beta_m\gamma$ coexpression and two components for $\alpha\beta\gamma\gamma_m$ coexpression. Figure 4 presents plots of the EC_{50} values versus the fraction of the wild-type component for each of the three coexpression experiments. The fraction of the wild-type component is the amplitude of the wild-type component divided by the total amplitude and is determined from the fits to the individual compound dose–response relationships. The EC_{50} values and component fractions are presented in Table 1. The relative fraction of the components varied in a manner that would be expected for the different cRNA injection ratios; that is, the amplitude of the wild-type component increased with an increase in the ratio of α-to-α_m cRNA. Figure 4*A* is a plot of the EC_{50} values of the three components versus the fraction of the wild-type component for $\alpha\alpha_m\beta\gamma$ coexpression. The dashed lines represent the mean EC_{50} determined from expression of $\alpha\beta\gamma$ and $\alpha_m\beta\gamma$ (Fig. 1*B*). The EC_{50} values of the first and third components do not depend on the relative proportions of the different receptor combinations consistent with the interpretation that they represent the activation of receptors containing all mutant and all wild-type α subunits, respectively. The continuous line is from a linear regression to the EC_{50} of the intermediate component and demonstrates a very slight, but significant ($p < 0.05$), negative dependence on the fraction of the wild-type component. That is, the EC_{50} decreased slightly with increasing availability of the wild-type α subunit. If the intermediate component was the combination of more than one subcomponent, the expected relationship should be in the opposite direction than that observed; that is, the EC_{50} should increase with an increase in the fraction of the wild-type component. The slight decrease in the EC_{50} with increasing fraction of the wild-type component may reflect differences in the ability to extract the EC_{50} values from these compound dose–response relationships as the amplitudes of the components vary. Figure 4*B,C* are similar plots of the EC_{50} values as a function of the fraction of the wild-type component with $\alpha\beta\beta_m\gamma$ and $\alpha\beta\gamma\gamma_m$ coexpression. In both cases, the EC_{50} values of all components appeared independent of the component amplitudes (Table 1). The amplitudes of the components in these wild-type and mutant coexpression experiments did not correspond in all cases to that predicted by the binomial hypothesis. Possible explanations for this discrepancy will be considered in the Discussion.

Nevertheless, the data presented in Figure 4 suggest that in terms of GABA sensitivity, there are three receptor types with $\alpha\alpha_m\beta\gamma$ and $\alpha\beta\beta_m\gamma$ coexpression and two receptor types with $\alpha\beta\gamma\gamma_m$ coexpression, further supporting a pentameric $GABA_A$ receptor composed of two α subunits, two β subunits, and one γ subunit.

The stoichiometry does not depend on the relative availability of α, β, and γ subunits

Finally, we considered whether the stoichiometry of the GABA receptor is fixed or can vary, depending on the relative abundance of the different subunit types. To address this issue, we injected α_m, β, and γ cRNA at a ratio of 4:1:1 or α, β, and γ_m cRNA at a ratio of 1:1:4. If the increased availability of α or γ subunits can increase the number of α or γ subunits incorporated into the GABA receptor, the EC_{50} values should shift further to the left than that observed with equivalent cRNA injection ratios (Fig. 1*B*). The results from these experiments are presented in Figure 5. For both cases, the EC_{50} values of the GABA dose–response relationships with nonequivalent injection ratios were indistinguishable from those determined with equivalent cRNA injection ratios. These data indicate that at least at these cRNA ratios, the number of α and γ subunits in the GABA receptor is fixed. By default, the number of β subunits should be fixed as well.

DISCUSSION

Summary, assumptions, and limitations

By taking advantage of a mutation in the putative second membrane-spanning domain of the $GABA_A$ receptor subunits that increased the GABA sensitivity in relative proportion to the number of subunits carrying the mutation, we were able to infer that recombinant $\alpha1\beta2\gamma2$ receptors are pentamers composed of two α subunits, two β subunits, and one γ subunit. We have also demonstrated that at least over a fourfold ratio of injected cRNA concentrations, the stoichiometry appears to be fixed. Behe et al. (1995) used a similar strategy of wild-type and mutant subunit coexpression to determine the number of incorporated NR1 sub-

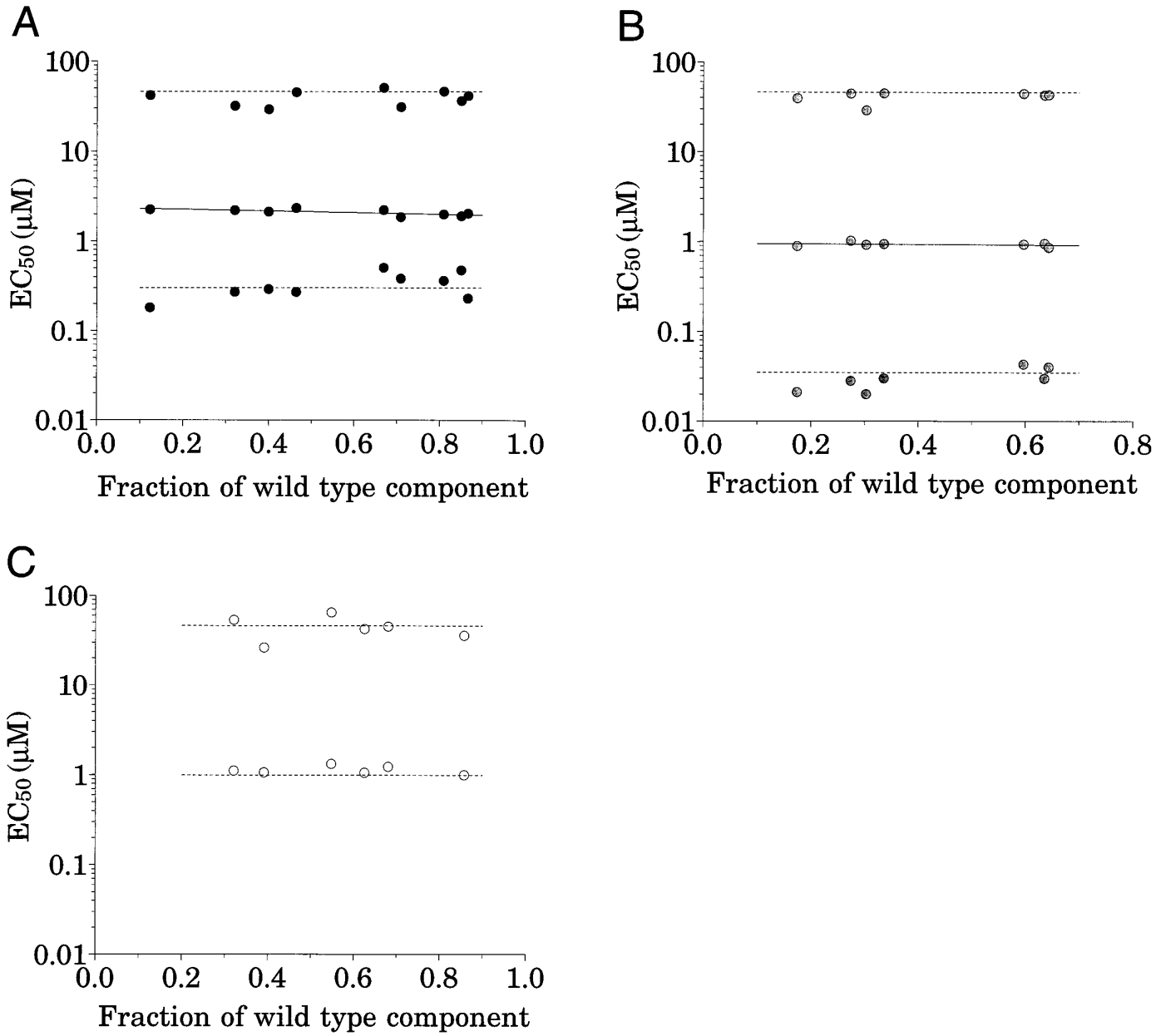

Figure 4. The EC_{50} values of the components with $\alpha\alpha_m\beta\gamma$, $\alpha\beta\beta_m\gamma$, and $\alpha\beta\gamma\gamma_m$ coexpression do not depend on the relative amplitudes of the components. *A*, Plot of the EC_{50} values of the three components as a function of the fraction of the wild-type component for $\alpha\alpha_m\beta\gamma$ coexpression. The fraction of the wild-type component is the amplitude of the wild-type component divided by the total amplitude and is determined from the fits to the individual compound dose–response relationships. In these experiments, GABA dose–response relationships were constructed from oocytes in which the α-to-α_m cRNA injection ratio was varied (1:3, 1:1, and 3:1) to shift the relative amplitudes of the different components. The total amount of α and α_m cRNA remained fixed with respect to β and γ. For all α-to-α_m ratios tested, the GABA dose–response relationships were described by the sum of three Hill equations. The *dashed lines* represent the mean EC_{50} values determined from $\alpha\beta\gamma$ and $\alpha_m\beta\gamma$ coexpression (Fig. 1). The *continuous line* is a linear regression to the EC_{50} of the intermediate component and demonstrates a slight but significant ($p < 0.05$) negative dependence that is in the opposite direction from that predicted if there were multiple, indistinguishable, intermediate components (see text). These data indicate three components and hence two α subunits in the GABA receptor complex. *B*, Similar plot as in *A*, but for $\alpha\beta\beta_m\gamma$ coexpression at different ratios of β to β_m (1:1 and 3:1). The *dashed lines* represent the mean EC_{50} values determined from $\alpha\beta\gamma$ and $\alpha\beta_m\gamma$ coexpression (Fig. 1). These data indicate three components and hence two β subunits in the GABA receptor complex. *C*, Similar plot as in *A*, but for $\alpha\beta\gamma\gamma_m$ coexpression at different ratios of γ to γ_m (1:3 and 1:1). These data indicate two components and hence one γ subunit in the GABA receptor complex.

units in recombinant *N*-methyl-D-aspartate (NMDA) receptors, although their approach differs from the present study in that they relied on mutation-induced alterations in the single-channel conductance.

Our conclusions are tied strongly to the assumption that the mutations do not alter the normal stoichiometry of the $\alpha1\beta2\gamma2$ $GABA_A$ receptor. Although we have no direct evidence, it seems unlikely that a point mutation in a residue that is presumed to face the interior of the pore (Revah et al., 1991; Unwin, 1995; Xu and Akabas, 1996) would so drastically affect subunit–subunit interactions as to induce an atypical stoichiometry. We have also assumed that the different possible subunit arrangements of the

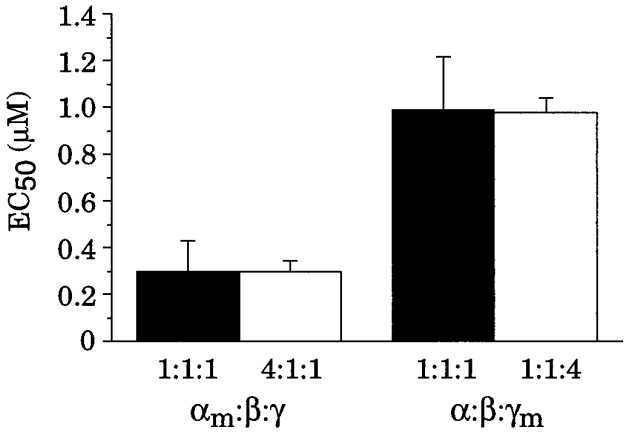

Figure 5. The stoichiometry does not depend on the $\alpha\beta\gamma$ cRNA injection ratio. The $\alpha_m\beta\gamma$ and $\alpha\beta\gamma_m$ cRNA combinations were injected into oocytes at ratios of 4:1:1 and 1:1:4, respectively. The GABA dose–response relationships were then fit by the Hill equation and compared with those determined with 1:1:1 cRNA injections. The EC_{50} values of the GABA dose–response relationships for $\alpha_m\beta\gamma$ cRNA ratios of 1:1:1 and 4:1:1 were (mean ± SD) 0.30 ± 0.13 μM (n = 9) and 0.30 ± 0.04 μM (n = 5), respectively. The EC_{50} values of the GABA dose–response relationships for $\alpha\beta\gamma_m$ cRNA ratios of 1:1:1 and 1:1:4 were 0.99 ± 0.23 μM (n = 4) and 0.98 ± 0.062 μM (n = 4), respectively. These data indicate that at least at these cRNA ratios, the number of α and γ subunits in the GABA receptor is fixed.

mutant and wild-type subunits within the pentameric complex are functionally equivalent.

To uncover any possible hidden components in the compound dose–response relationships of Figure 3, we varied the wild-type-to-mutant cRNA ratio for each of the subunits (e.g., α, α_m) and coexpressed this mixture along with their wild-type counterparts (e.g., β, γ). Although the relative amplitudes of the components depended on the wild-type-to-mutant cRNA ratio, the observed number of components and their EC_{50} values were essentially independent of the cRNA ratio. This supports the conclusion that the dose–response relationships in Figure 3*A–C* are composed of only three, three, and two components, respectively.

We do not believe that the shifts in EC_{50} in Figure 1, and the additional components in Figure 2, result from the expression of subpopulations of GABA receptors with different GABA sensitivities (e.g., $\alpha\beta$, $\beta\gamma$, etc.) for the following reasons. (1) Expression of α, β, or γ subunits alone, as well as α/γ coexpression, does not yield functional GABA receptors in oocytes. (2) $\beta\gamma$ receptors have an EC_{50} of 30.1 ± 8.4 μM, which is similar to that of $\alpha\beta\gamma$ GABA receptors (45.8 ± 3.6 μM). (3) $\alpha\beta$ GABA receptors have an EC_{50} of 4.2 ± 0.9 μM (Amin et al., 1994), which is less GABA-sensitive than any of the nonwild-type components (Figs. 2 and 3; Table 1). The observation that the EC_{50} values do not shift with changes in the wild-type-to-mutant coinjection ratios (Fig. 4; Table 1) further strengthens the conclusion that subpopulations of GABA receptors cannot account for the shifts in GABA sensitivity.

The amplitudes of the components for the different cRNA injection ratios deviated somewhat from that predicted by the binomial distribution. For example, at a wild-type-to-mutant cRNA ratio of 1:1 (Table 1), the fractional amplitudes of the wild-type components were 0.72 ± 0.07 (n = 3), 0.27 ± 0.07 (n = 4), and 0.72 ± 0.12 (n = 3) for $\alpha\alpha_m\beta\gamma$, $\alpha\beta\beta_m\gamma$, and $\alpha\beta\gamma\gamma_m$, respectively. Assuming that the percentages of the different receptor combinations were to follow a binomial distribution, the fraction of wild-type receptors should be 0.25 for $\alpha\alpha_m\beta\gamma$ and $\alpha\beta\beta_m\gamma$ coexpression and 0.50 for $\alpha\beta\gamma\gamma_m$ coexpression. Several factors could account for this discrepancy. First, the mutations may affect subunit assembly. If the mutant subunit does not assemble as efficiently as that of the wild type, the amplitudes of the components would deviate from those predicted by the binomial distribution. Second, if the mutation altered the maximum percent time open or the single-channel conductance, the relative amplitudes of the components would be altered accordingly. In fact, the mutations do affect the Hill coefficients slightly (see Fig. 1 legend), suggesting an alteration in the gating kinetics. Third, the determination of the amplitudes of the components may be influenced by receptor desensitization. At high agonist concentrations, desensitization would diminish the relative contribution of the receptors in the more GABA-sensitive components, thus depressing the apparent amplitude of the wild-type component. Fourth, in the case of $\alpha_m\beta\gamma$ and $\alpha\beta_m\gamma$ coexpression, we observed a picrotoxin-sensitive current in the absence of GABA that was not observed in oocytes expressing $\alpha\beta\gamma_m$ or $\alpha\beta\gamma$ receptors (data not shown). This may represent the spontaneous opening of GABA receptors with two mutant subunits incorporated. If so, this would alter the relative contributions of the components in the compound dose–response relationship with $\alpha\alpha_m\beta\gamma$ and $\alpha\beta\beta_m\gamma$ coexpression. And finally, there may be slight errors in estimating the relative wild-type and mutant cRNA concentrations from the agarose gels. Although these factors could influence the estimation of the parameters in these compound dose–response relationships, it is not apparent how they would alter the general conclusions from this study, which rely more heavily on a detection of the number of components. Moreover, our conclusions are strengthened by the excellent agreement between the observed and predicted EC_{50} values of the components in Figures 3 and 4.

Comparison with other studies

Our studies are consistent with evidence indicating that some native (Duggan et al., 1991; Luddens et al., 1991; Pollard et al., 1995) and recombinant (Verdoorn, 1994) GABA receptors can contain multiple subtypes of the α subunit. In addition, Im et al. (1995), using tandem constructs of α6-β2 GABA subunits, concluded that $\alpha6\beta2\gamma2$ GABA receptors contain two α subunits, two β subunits, and one γ subunit.

Our results are in contrast to those of Benke et al. (1994), which infer that only one β subunit is present in the native GABA receptor. This conclusion was reached from the observation that [^{3}H]flumazenil or [^{3}H]muscimol binding to native GABA receptors immunoprecipitated from brain by β1, β2, or β3 antiserum sum to ~100% of the total binding. If neurons dominantly express one isoform of the β subunit, however, the results of Benke et al. (1994) would be obtained regardless of the number of β subunits in the GABA receptor complex. In fact, immunolocalization studies indicate some segregation of the β subunit isoforms to different brain regions (Benke et al., 1994).

Quirk et al. (1994) observed that the sum of the percentages of [^{3}H]muscimol binding sites immunoprecipitated by γ2 and γ3 subunit antisera was slightly greater than the [^{3}H]muscimol binding observed with γ2 and γ3 antisera in combination, although as the authors report, the difference was within the limit of error of the measurement. Khan et al. (1994) made a similar observation using antibodies raised to γ2S and γ2L subunits. In both cases, the data were used to conclude that a minor fraction of the GABA receptors can contain more than one type of γ subunit. Further-

more, GABA receptors obtained by γ3 immunoprecipitation were labeled in a Western blot probed with γ2 antisera, again suggesting that γ2 and γ3 subunits can coexist in the same GABA receptor complex. This conclusion seems to contradict our findings that the α1β2γ2 GABA receptor contains a single copy of the γ2 subunit. It should be kept in mind, however, that immunoprecipitation studies investigate total GABA or benzodiazepine binding to isolated membranes. This approach may include (1) a subpopulation of nonfunctional GABA receptors having an aberrant stoichiometry (our approach is limited to functional $GABA_A$ receptors) or (2) a subpopulation of βγ or αγ GABA receptors that may contain multiple γ subunits. It is also possible that GABA receptors may have a stoichiometry that depends on the expression system (neurons, transfected mammalian cells, oocytes) or the particular α, β, and γ subunit isoforms.

Finally, Backus et al. (1993) examined the stoichiometry of rat α3β2γ2 receptors in transfected human embryonic kidney cells (HEK293) and concluded that the possible subunit stoichiometries for this receptor were 2α1β2γ, 2α2β1γ, or 1α2β2γ, of which 2α1β2γ was favored. This study examined the degree of outward rectification induced by charged substitutions in homologous positions of the putative pore-forming domain of the α3, β2, and γ2 subunit isoforms. As stated by the authors, the conclusions assumed that the degree of the effect of changing the charge on the outward rectification is the same for all the subunits. Given that the amino acid at one of the two mutated positions was not conserved across the three subunits (arginine, tyrosine, or lysine), this assumption may not hold true. Perhaps both conservative and nonconservative substitutions (in terms of charge) at these positions, along with wild-type and mutant coexpression of each subunit type, could be used to test this assumption.

Arrangement of the subunits

The question of the precise manner in which the two α subunits, two β subunits, and one γ subunit are arranged within the pentameric structure is still unanswered. Some insight may be provided by structure/function studies of the $GABA_A$ receptor. For example, site-directed mutagenesis has previously identified a residue on the α subunit and two domains on the β subunit that seem to form part of the GABA binding site (Sigel et al., 1992; Amin and Weiss, 1993). If, as for the nACh receptor, the agonist binding sites are at subunit interfaces (Kurosaki et al., 1987; Blount and Merlie, 1989; Pedersen and Cohen, 1990; Czajkowski and Karlin, 1991; Sine and Claudio, 1991; Sine, 1993), at least one of the two GABA binding sites may be at the α–β subunit interface (Smith and Olsen, 1995). A detailed single-channel kinetic analysis (Sine and Steinbach, 1987; Colquhoun and Ogden, 1988; Weiss and Magleby, 1989; Twyman et al., 1990; Newland et al., 1991) may provide information on the equivalence of the two GABA binding sites and help place the second binding site at either the other α-β interface or at a β-γ or α-γ interface.

Although the potential subunit interactions underlying activation and modulation of $GABA_A$ receptors are yet to be elucidated, knowledge of the stoichiometry will facilitate the incorporation of the wealth of structural and functional information into a cohesive model of the $GABA_A$ receptor/pore complex.

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