

A neurosteroid potentiation site can be moved among GABA_A receptor subunits

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Key points

- Neuroactive steroids are endogenous compounds that are potent and efficacious potentiators of GABA_A receptors.
- Neuroactive steroids have been shown to act after interacting with amino acids in the first transmembrane-spanning region (TM1 region) of the α subunit.
- We show that the TM1 region can be moved from the α 1 subunit to other subunits of the GABA_A receptor (the β 2 or γ 2 subunits) and confer potentiation.
- Our data show that neurosteroid potentiation does not require that the steroid interacts with a particular subunit of the GABA_A receptor, nor that it interacts with a subunit that also binds GABA.
- Our data also indicate that a steroid binding site is located in the TM1 region.

Abstract Endogenous neurosteroids are among the most potent and efficacious potentiators of activation of GABA_A receptors. It has been proposed that a conserved glutamine residue in the first membrane-spanning region (TM1 region) of the α subunits is required for binding of potentiating neurosteroids. Mutations of this residue can reduce or remove the ability of steroids to potentiate function. However, it is not known whether potentiation requires that a steroid interact with the α subunit, or not. To examine this question we mutated the homologous residue in the β 2 and γ 2L subunits to glutamine, and found that these mutations could not confer potentiation by allopregnanolone (3 α 5 α P) when expressed in receptors containing ineffective α 1 subunits. However, potentiation is restored when the entire TM1 region from the α 1 subunit is transferred to the β 2 or γ 2L subunit. Mutations in the TM1 region that affect potentiation when made in the α 1 subunit have similar effects when made in transferred TM1 region. Further, the effects of 3 α 5 α P on single-channel kinetics are similar for wild-type receptors and receptors with moved TM1 regions. These results support the idea that steroids bind in the transmembrane regions of the receptor. The observations are consistent with previous work indicating that neurosteroid potentiation is mediated by an action that affects the receptor as a whole, rather than an individual subunit or pair of subunits, and in addition demonstrate that the mechanism is independent of the nature of the subunit that interacts with steroid.

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Abbreviations 3 α 5 α P, (3 α , 5 α)-3-hydroxypregnan-20-one, allopregnanolone; P4S, piperidine-4-sulfonic acid; TM1 region, first membrane-spanning region.

Introduction

Endogenous neurosteroids are among the most potent and efficacious potentiators of activation of GABA_A receptors. Steroids act on these receptors in regions exposed to the cell membrane (Akk *et al.* 2005), and a particular residue in the first membrane-spanning region (TM1 region) of the $\alpha 1$ subunit ($\alpha 1$ Gln241) has been shown to be required for potentiation (Hosie *et al.* 2006). Examination of a series of mutations and of steroids of different structures resulted in the proposal that this residue is directly involved in steroid binding (Hosie *et al.* 2006), although further studies have demonstrated that the interactions between a potentiating steroid and the receptor are likely to be complex (Akk *et al.* 2008; Li *et al.* 2009). All GABA_A receptor α subunits have a glutamine residue at this position, and mutation of this glutamine to leucine in other α subunits also removes neurosteroid potentiation from receptors containing the mutated subunit (Hosie *et al.* 2009). These observations suggest that steroid potentiation requires the presence of this residue in the α subunit.

To test this idea, and to explore the role of homologous regions in other subunits, we mutated the corresponding residue in the $\beta 2$ and $\gamma 2L$ subunits (tryptophan in both cases) to glutamine, and found that potentiation was not restored for receptors containing a mutated (steroid-insensitive) $\alpha 1$ subunit. However, a study of potentiation of the nicotinic $\alpha 4\beta 2$ receptor by estradiol (Jin & Steinbach, 2011) raised the possibility that a larger region might be required. Indeed, we find that when the entire TM1 region is transplanted from the $\alpha 1$ subunit to the $\beta 2$ or $\gamma 2L$ subunit, steroid potentiation is restored in receptors with steroid-insensitive $\alpha 1$ subunits.

Methods

Receptors were expressed using GABA_A receptor subunits from rat: $\alpha 1$ (NCBI Reference Sequence: NP_899155.1); $\beta 2$ (NP_037089.1); and $\gamma 2L$ (NP_899156.1). Subunits were inserted in the expression vector pcDNA3 (Invitrogen, San Diego, CA, USA). HEK cells were plated at a density of 200,000 cells dish⁻¹ 1 day before transfection. A total of 0.4 μ g of plasmid cDNAs at 1:1:1 ratio was mixed with 10 μ l of Effectene reagent and 3.2 μ l of Enhancer of Effectene (QIAGEN, Valencia, CA, USA) per dish. Fifteen–twenty hours later, cells were washed with culture medium. Recordings were made 1–3 days after transfection.

The amino acid residues in the first transmembrane regions for the three subunits are shown in Fig. 1. The polynucleotide sequence coding for the entire region shown was transferred; that is, the residues shown for the $\alpha 1$ sequence were substituted for the residues shown for the $\beta 2$ or $\gamma 2L$ sequence. Point mutations were made using QuikChange site directed mutagenesis (Stratagene, San

Diego, CA, USA). The entire coding sequence for all constructs was sequenced to ascertain that only the desired mutations were made.

3 α 5 α P ((3 α , 5 α)-3-hydroxypregnan-20-one, allopregnanolone) was purchased from Sigma-Aldrich (St Louis, MO, USA) and prepared as a 10 mM stock solution in DMSO and diluted on the day of the experiment. Concentrations of steroid above 10 μ M were not used, as the steroid solubility is not known precisely but is likely to be less than 30 μ M.

The $\alpha 1$ subunit is epitope (FLAG)-tagged (Ueno *et al.* 1996) in the amino-terminal end of the subunit. Cells expressing high levels of surface receptors were selected for physiological recordings using a bead-binding technique in which the presence of the FLAG peptide was detected with a mouse monoclonal antibody to the FLAG epitope (M2; Sigma-Aldrich), which had been adsorbed to beads with a covalently attached goat anti-mouse IgG antibody (Invitrogen).

Whole-cell patch-clamp records were obtained and analysed as described (Li *et al.* 2006). To characterize activation by GABA, six–eight concentrations of GABA were applied and the peak response measured. Normalized concentration–response curves for each cell were fit with the Hill equation to estimate the [GABA] producing a half-maximal response (EC₅₀). Potentiation was assessed by the ratio of the response of a cell to a low concentration of GABA + steroid to the response of that cell to the same concentration of GABA in the absence of steroid (the ‘potentiation ratio’). Concentrations of GABA were selected to produce ~15% of the maximal response, from the concentration–response relationship for that particular combination of subunits. Direct activation by steroid was assessed by determining the response of a cell to the application of 10 μ M steroid in the absence of GABA, and normalized to the response of the same cell to 1 mM GABA. This ratio is essentially the fraction of maximal activation that 10 μ M steroid can elicit. Two statistical tests were made of the potentiation ratio. In the first, the ratios for a given construct and condition (e.g. wild-type receptors using 1 μ M 3 α 5 α P) were compared with 1 (no effect) using a paired *t* test. This test indicated whether a significant effect was seen. In the second, potentiation ratios were compared across sets of constructs using one-way ANOVA with a Dunnett *post hoc* correction. This test indicated whether responses of a given construct differed from the base construct in the set.

Single-channel recordings were made in the cell-attached mode, and analysed as described previously (Akk & Steinbach, 2000; Akk *et al.* 2008). In the single-channel recordings of cells expressing $\alpha 1$ (I&L) + $\beta 2$ (TM1) + $\gamma 2L$ subunits we observed some clusters of activity in the presence of 3 α 5 α P that appeared to be unaffected by 3 α 5 α P. These clusters were assumed to result from incorporation

Table 1. Effects of mutations on potentiation and gating by $3\alpha 5\alpha P$

Subunits	Potentiation by $0.1 \mu M 3\alpha 5\alpha P$			Potentiation by $1 \mu M 3\alpha 5\alpha P$			Potentiation by $10 \mu M 3\alpha 5\alpha P$			Gating by $10 \mu M 3\alpha 5\alpha P$		
	mean \pm SEM (N)	P to 1	P in set	mean \pm SEM (N)	P to 1	P in set	mean \pm SEM (N)	P to 1	P in set	mean \pm SEM (N)	P to 0	P to wt
$\alpha 1 \beta 2 \gamma 2L$	2.67 \pm 0.26 (5)	**		4.98 \pm 0.61 (10)	***		4.81 \pm 0.42 (5)	***		0.034 \pm 0.009 (10)	**	—
$\alpha 1(I\&L) \beta 2 \gamma 2L$	ND			1.19 \pm 0.15 (7)	NS		1.25 \pm 0.06 (6)	**	—	0.001 \pm 0.001 (13)	NS	***
$\alpha 1(I\&L) \beta 2(TM1) \gamma 2L$	2.18 \pm 0.25 (5)	**		3.39 \pm 0.30 (11)	***	***	2.53 \pm 0.23 (11)	***	***	0.003 \pm 0.002 (7)	NS	***
$\alpha 1(I\&L) \beta 2 \gamma 2L(TM1)$	1.57 \pm 0.18 (6)	*		2.08 \pm 0.16 (13)	***	*	2.91 \pm 0.26 (6)	***	***	0.007 \pm 0.004 (7)	NS	**
$\alpha 1(I\&L) \beta 2(W237Q)$	ND			1.01 \pm 0.03 (5)	NS	NS	ND			0.003 \pm 0.002 (5)	NS	**
$\gamma 2L$												
$\alpha 1(I\&L)$	ND			1.03 \pm 0.09 (6)	NS	NS	ND			0.003 \pm 0.002 (6)	NS	***
$\beta 2(I232T\&W237Q)$												
$\gamma 2L$												
$\alpha 1(I\&L) \beta 2$	ND			0.92 \pm 0.05 (5)	NS	NS	ND			0.008 \pm 0.004 (5)	NS	**
$\gamma 2L(W252Q)$												
$\alpha 1(I\&L) \beta 2(TM1$	ND			ND			1.06 \pm 0.04 (15)	NS	NS	0.001 \pm 0.001 (4)	NS	**
$Q237L) \gamma 2L$												
$\alpha 1(I\&L) \beta 2(CTVQ) \gamma 2L$	ND			2.37 \pm 0.13 (7)	***	**	ND			0.001 \pm 0.000 (5)	NS	***
$\alpha 1(I\&L) \beta 2(TM1$	ND			ND			1.12 \pm 0.14 (11)	NS	NS	0.004 \pm 0.004 (5)	NS	**
$W241L) \gamma 2L$												
$\alpha 1(W245L) \beta 2 \gamma 2L$	ND			1.04 \pm 0.10 (5)	NS	—	ND			0.001 \pm 0.001 (5)	NS	***
$\alpha 1(W245L) \beta 2(TM1)$	ND			2.18 \pm 0.15 (5)	**	***	ND			0.004 \pm 0.001 (5)	*	**
$\gamma 2L$												
$\alpha 1(W245L) \beta 2$	ND			1.14 \pm 0.16 (8)	NS	NS	ND			0.001 \pm 0.000 (8)	**	***
$\gamma 2(TM1)$												

The first column presents the subunits transfected. The first group of columns first shows the potentiation ratio for data from cells tested with a GABA concentration producing 15–20% of the maximal response $\pm 0.1 \mu M 3\alpha 5\alpha P$. The column headed 'P to 1' summarizes the probability that the ratio is the same as a value of 1 (no potentiation; paired two-tailed *t* test). 'P in set' summarizes the probability that the ratio is the same as that for receptors in the first row of the set (e.g. $\alpha 1(I\&L) \beta 2 \gamma 2L$; one-way ANOVA with Dunnett correction). The next two groups of columns show similar data for potentiation by 1 and 10 $\mu M 3\alpha 5\alpha P$. The final group of columns shows data for direct gating by 10 $\mu M 3\alpha 5\alpha P$ in the absence of GABA, normalized to the response to 1 mM GABA in the same cell (i.e. fraction of maximal GABA-elicited response). 'P to 0' summarizes the probability that the ratio is equal to 0 (no gating; two-tailed paired *t* test), and 'P to wt' the probability that the value is the same as that for receptors containing wild-type subunits (first row; one-way ANOVA with Dunnett correction). Probabilities summarized as: NS: $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. ND: experiment not done. All data are presented as mean \pm SEM (N cells).

solubility. As can be seen, there were only small increases in potentiation between 1 and 10 μM steroid, which is consistent with previous studies that indicate 1 μM is a saturating concentration. For $\alpha 1 + \beta 2 + \gamma 2L$ and $\alpha 1(I\&L) + \beta 2(TM1) + \gamma 2L$ receptors, the potentiation ratio did not differ at 10 μM from 1 μM ; while for $\alpha 1(I\&L) + \beta 2 + \gamma 2L(TM1)$ receptors the increase was significant ($P < 0.01$, two-tailed *t* test).

Inspection of the region transferred (see Fig. 1) shows a total of eight differences between $\beta 2$ and $\alpha 1$ in this region. We mutated both $\beta 2(I231T)$ and $\beta 2(W237Q)$ as these residues were implicated in steroid actions (Hosie *et al.* 2006). However, this did not confer potentiation when expressed with $\alpha 1(I\&L)$ and $\gamma 2L$ (Table 1). Among the eight differing residues, four are differences that occur relatively rarely in related proteins (as assessed from the BLOSUM62 matrix; Henikoff & Henikoff, 1992). These are the pairs $\beta 2(S229)/\alpha 1(C233)$, $\beta 2(I232)/\alpha 1(T236)$, $\beta 2(T233)/\alpha 1(V237)$ and $\beta 2(W237)/\alpha 1(Q241)$. Accordingly, we tested the consequences of mutating all four of these residues in the $\beta 2$ TM1 region ($\beta 2(CTVQ)$),

and found that this reduced number of mutations was capable of conferring potentiation (Table 1; Fig. 3). This observation indicates that the entire TM1 region is not required, but further work is necessary to define the roles of amino acids in underlying steroid potentiation.

Consequences of mutations

We then examined whether the transferred TM1 regions showed some properties for potentiation that are similar to those when the region is located in the $\alpha 1$ subunit. In the first case, we mutated the critical glutamine in the transferred TM1 region to leucine. This removed potentiation: $\beta 2(TM1 Q237L)$ had a potentiation ratio of 1.06 ± 0.04 (15) when GABA was applied with 10 $\mu M 3\alpha 5\alpha P$, which did not differ from no effect (Table 1).

We also mutated a tryptophan residue in the $\alpha 1$ TM1 region ($\alpha 1(W245L)$), that we have previously shown to remove potentiation by neurosteroids (Akk

et al. 2008; see Fig. 1 for position). We confirmed that expression of $\alpha 1$ (W245L) with wild-type $\beta 2$ and $\gamma 2L$ subunits resulted in receptors that were not potentiated by $3\alpha 5\alpha P$ (Fig. 3; Table 1). However, expression of $\alpha 1$ (W245L) with $\beta 2$ (TM1) and wild-type $\gamma 2L$ did result in significant potentiation of the receptors (Fig. 3; Table 1), although expression with $\beta 2 + \gamma 2L$ (TM1) did not. The $\alpha 1$ (W245L) mutation reduced the amount of potentiation compared with the $\alpha 1$ (I&L) mutation ($P < 0.01$ for either the $\beta 2$ (TM1) + $\gamma 2L$ or $\beta 2 + \gamma 2L$ (TM1) constructs; two-tailed *t* test). We do not have an explanation for this difference between the effects of the two $\alpha 1$ mutations. The inverse experiment, mutating the transferred tryptophan in $\beta 2$ ($\beta 2$ (TM1 W241L)) abolished potentiation when expressed with $\alpha 1$ (I&L) (Table 1). These results demonstrate that critical residues in the transferred TM1 regions have roles similar to those in the $\alpha 1$ TM1 region.

Single-channel kinetics

One possibility is that steroid potentiation of receptors with transferred TM1 regions occurs by a different kinetic mechanism than for wild-type receptors. To examine this, we performed cell-attached single-channel recordings of activity elicited by $100 \mu M$ GABA in the

absence and presence of $10 \mu M$ $3\alpha 5\alpha P$, for receptors comprising $\alpha 1$ (I&L) + $\beta 2$ (TM1) + $\gamma 2L$ subunits. In the absence of $3\alpha 5\alpha P$ the open time distributions contained only two components (Fig. 4; Table 2), rather than the three components seen in wild-type receptors activated by GABA (Akk *et al.* 2008). We have already reported that receptors composed of $\alpha 1$ (Q241L) with wild-type $\beta 2$ and $\gamma 2L$ subunits also show only two open time components (Akk *et al.* 2008), so it is likely that this observation results from a dominant kinetic effect of the $\alpha 1$ Q241L mutation. In the presence of $3\alpha 5\alpha P$, three components were seen in the open time distributions. The duration of the second component was prolonged, and a new longer duration component appeared (Fig. 3; Table 2). Similarly, the longest intracluster closed time was greatly reduced in prevalence in the presence of $3\alpha 5\alpha P$ (Fig. 4; Table 2). These changes are similar to wild-type receptors potentiated by $3\alpha 5\alpha P$, for which there is an increase in both the prevalence and duration of the longest duration open time component, and a reduction in the prevalence of the longest intracluster closed time (Akk *et al.* 2004). It is interesting to note that wild-type receptors activated by piperidine-4-sulfonic acid (P4S), a low efficacy agonist, show only two components in the open time distributions in the absence of potentiating neurosteroid, similar to $\alpha 1$ (Q241L)-containing receptors activated by GABA (Akk

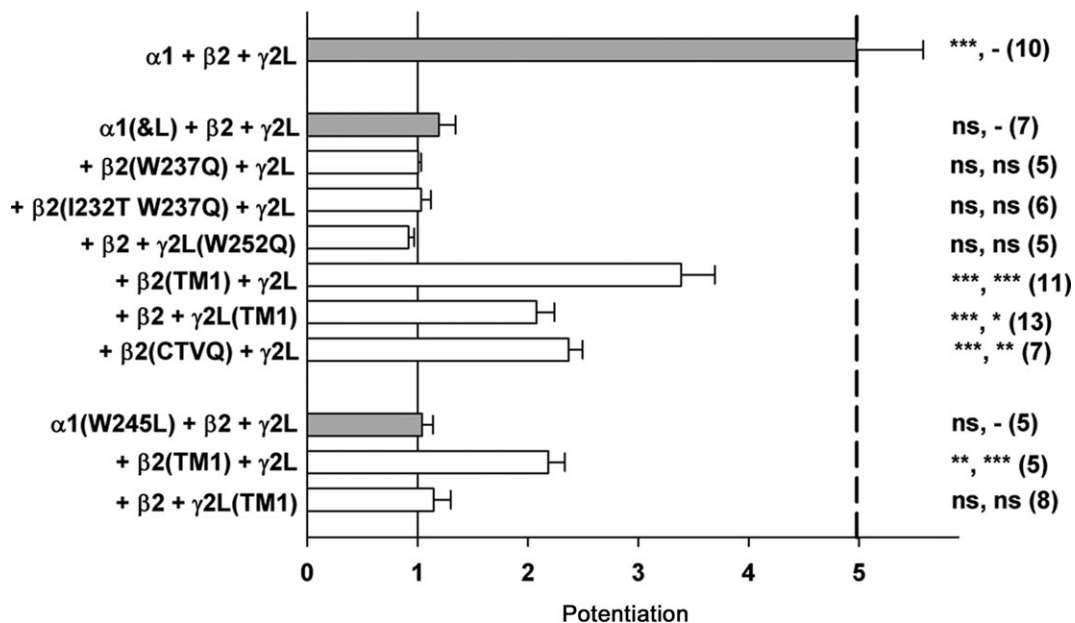


Figure 3. Summary of potentiation of GABA responses

The mean (\pm SEM) potentiation by $1 \mu M$ $3\alpha 5\alpha P$ is shown. The combinations of subunits are grouped as follows: top combination is all wild-type subunits; the next group all contain $\alpha 1$ (I&L) with various constructs; and the bottom group all contain $\alpha 1$ (W245L). The thin vertical continuous line shows a value of 1 (no potentiation), and the thick dashed line shows the mean potentiation for wild-type receptors. The symbols to the right of the bars indicate probability that potentiation differs significantly from 1 (that is no effect; two-tailed paired *t* test) and from the potentiation from the initial combination in the group (e.g. $\alpha 1$ (I&L) + $\beta 2 + \gamma 2L$; one-way ANOVA with Dunnett's *post hoc* correction) (initial combination, NS $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Data values are presented in Table 1.

et al. 2008). When wild-type receptors activated by P4S are exposed to $3\alpha5\alpha\text{P}$, a third open time component appears and the prevalence of the long duration closed component is reduced (Akk *et al.* 2008), as we see with GABA activation of $\alpha1(\text{Q241L})$ -containing receptors expressed with potentiation-conferring subunits. In sum, the kinetic mechanisms by which neurosteroids potentiate receptors containing transferred TM1 regions appear indistinguishable from those for wild-type receptors.

Direct activation by $3\alpha5\alpha\text{P}$

Although potentiation by $3\alpha5\alpha\text{P}$ was restored, direct activation was not restored by any of the constructs when expressed with the $\alpha1(\text{I\&L})$ subunit (Table 1). Direct activation was tested with $10\ \mu\text{M}$ $3\alpha5\alpha\text{P}$, to use a high

concentration but within the aqueous solubility of the steroid. We note that direct gating is quite low in our hands, even for the wild-type receptor, so it is possible that potency, rather than efficacy, is affected. In any case, the absence of direct gating might result from the fact that activation has been proposed to involve bridging of a steroid between two adjacent subunits (Hosie *et al.* 2006), which might be abrogated by the altered neighbour relationships.

Effects of mutations on other receptor properties

We note that the TM1 region also appears to be important for activation of receptors by GABA. As found earlier (Hosie *et al.* 2006; Akk *et al.* 2008), the concentration of GABA required for half-maximal activation (EC_{50})

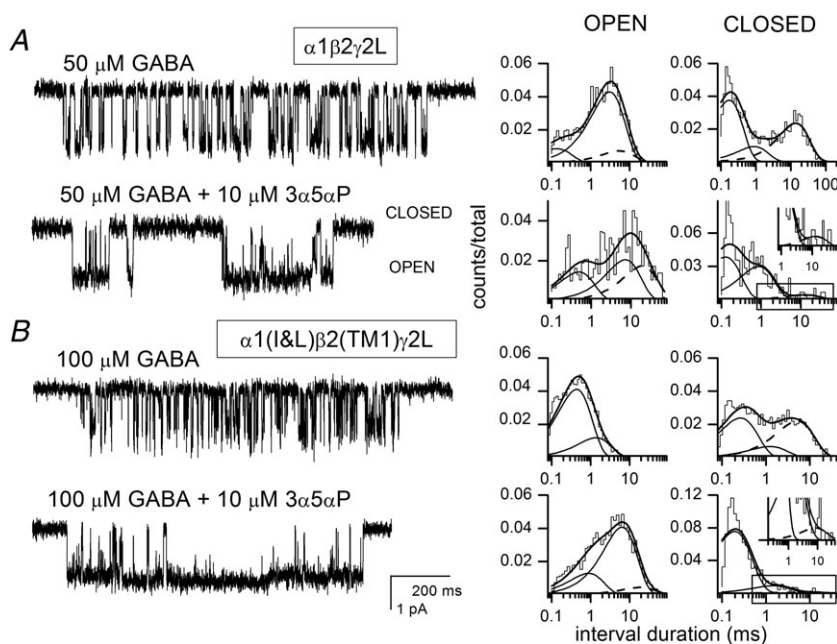


Figure 4. Potentiation of single-channel currents

Sample single-channel clusters from cell-attached patches from HEK cells expressing rat $\alpha1 + \beta2 + \gamma2\text{L}$ (A) or $\alpha1(\text{I\&L}) + \beta2(\text{TM1}) + \gamma2\text{L}$ receptors (B). The receptors were activated by $50\ \mu\text{M}$ or $100\ \mu\text{M}$ GABA, in the absence and presence of $10\ \mu\text{M}$ $3\alpha5\alpha\text{P}$. Openings are shown as downward deflections. The scale bars in A (1 pA, 200 ms) apply to A and B. The open and closed time histograms from the respective patches are shown next to the data traces. The lines show the fits to the data: the thick lines show the sum of all components fit; individual components are shown with thin continuous lines; while the dashed lines show the components discussed in the Results (OT3 and CT3). A, for GABA alone, the mean open times are 0.1 ms (15%), 2.8 ms (73%) and 5.2 ms (12%), and the mean closed times are 0.2 ms (55%), 0.8 ms (13%) and 12.7 ms (32%). For GABA + $3\alpha5\alpha\text{P}$, the mean open times are 0.5 ms (29%), 6.6 ms (40%) and 17.9 ms (31%), and the mean closed times are 0.1 ms (53%), 0.8 ms (41%) and 12.2 ms (6%). B, for GABA alone, the open times were fit with two components, with mean durations of 0.4 ms (79%) and 1.3 ms, and the closed times with three components of 0.2 ms (49%), 1.2 ms (13%) and 4.6 ms. For GABA + $3\alpha5\alpha\text{P}$, the open times required three components of 0.8 ms (26%), 5.3 ms (68%) and 17.2 ms (6%), and the closed times are 0.2 ms (88%), 1.4 ms (11%) and 7.9 ms (1%). $3\alpha5\alpha\text{P}$ enhances the mean open duration of the mutant receptor by introducing a new, long-lived open state (dashed line) and prolonging the mean duration of the second open time component. $3\alpha5\alpha\text{P}$ also acts on the closed time distribution by decreasing the prevalence of the longest-lived closed time component (dashed line). The inset in the closed time distribution in the presence of $3\alpha5\alpha\text{P}$ shows a magnified view of the longest duration closed time component (boxed in main histogram), to demonstrate the very small component. The mean values for kinetic parameters are shown in Table 2.

Table 2. Properties of single-channel activity from cells transfected with $\alpha 1 + \beta 2 + \gamma 2L$ (WT) or $\alpha 1(I&L) + \beta 2(TM1) + \gamma 2L$ subunits (mutant)

Receptor	GABA	3 α 5 α P	OT1	Fr OT1	OT2	Fr OT2	OT3	Fr OT3	N
WT	50		0.31 ± 0.14	0.20 ± 0.05	3.0 ± 0.5	0.67 ± 0.06	6.3 ± 2.6	0.13 ± 0.05	7
WT	50	10	0.29 ± 0.13 NS	0.29 ± 0.05*	3.0 ± 2.6 NS	0.31 ± 0.11***	16.5 ± 6.2**	0.40 ± 0.09***	4
mutant	100		0.39 ± 0.03	0.80 ± 0.07	1.2 ± 0.1	0.20 ± 0.07	—	0	4
mutant	100	10	0.53 ± 0.32 NS	0.24 ± 0.07***	5.5 ± 0.5**	0.61 ± 0.10**	27.8 ± 10.7	0.15 ± 0.08**	3

Receptor	GABA	AP	CT1	Fr CT1	CT2	Fr CT2	CT3	Fr CT3	N
WT	50		0.19 ± 0.10	0.56 ± 0.09	1.5 ± 0.6	0.15 ± 0.07	13.0 ± 3.5	0.29 ± 0.06	7
WT	50	10	0.12 ± 0.01 NS	0.55 ± 0.07 NS	1.0 ± 0.3 NS	0.35 ± 0.07**	10.7 ± 3.5 NS	0.10 ± 0.04***	4
mutant	100		0.24 ± 0.05	0.50 ± 0.06	1.3 ± 0.5	0.14 ± 0.02	6.2 ± 2.3	0.37 ± 0.04	4
mutant	100	10	0.18 ± 0.04 NS	0.81 ± 0.09*	1.2 ± 0.4 NS	0.16 ± 0.07 NS	11.2 ± 6.1 NS	0.04 ± 0.03***	3

Single-channel activity in clusters of openings was analysed to determine the numbers of components present, and their mean durations and overall fraction of the total events. Mean values are presented for the mean open time durations (OT1, OT2 and OT3) and the fractions of the total number of openings in each component. The longest duration component (OT3) was not present for activity from the mutant receptor in the absence of 3 α 5 α P, as is also true for receptors including the $\alpha 1(Q241L)$ subunit (Akk *et al.* 2008), but appeared in the presence of 3 α 5 α P. The longest duration closed time component (CT3) was significantly less common in the presence of 3 α 5 α P. (Significance of the difference between control and +3 α 5 α P by two-tailed *t* test for each receptor type; NS *P* > 0.05, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.) We compared the ability of kinetic models to describe the data by comparing the log-likelihoods for the respective fits (Horn, 1987). We fit open time distributions with either 2 or 3 open states, for both control (no 3 α 5 α P) and plus steroid for responses from $\alpha 1(I&L) + \beta 2(TM1) + \gamma 2L$ receptors. The differences in log-likelihood values for control recordings were 0, 4, 2 and 1 unit, while for recordings plus steroid they were 10, 11 and 16. For an assumed probability of 0.05 that the increased log-likelihood would arise by chance, the difference in log-likelihood for each added state should be greater than 3. We conclude that a 3 open state model does not provide a significantly better description for control records, but does for recordings in the presence of steroid.

Table 3. Effects of mutations on receptor properties

Subunits	EC ₅₀ for GABA activation (μ M)			Response to 1 mM GABA (–60 mV, pA)		
	mean ± SEM (N)	<i>P</i> to wt	<i>P</i> in set	mean ± SEM (N)	<i>P</i> to wt	<i>P</i> in set
$\alpha 1 \beta 2 \gamma 2L$	8 ± 2 (11)	—	—	–1908 ± 205 (22)	—	—
$\alpha 1(I&L) \beta 2 \gamma 2L$	270 ± 47 (6)	***	—	–976 ± 202 (18)	*	—
$\alpha 1(I&L) \beta 2(TM1) \gamma 2L$	34 ± 8 (4)	NS	*	–2259 ± 395 (21)	NS	**
$\alpha 1(I&L) \beta 2 \gamma 2L(TM1)$	356 ± 108 (6)	***	NS	–1371 ± 322 (13)	NS	NS
$\alpha 1(I&L) \beta 2(W237Q) \gamma 2L$	102 ± 20 (6)	NS	NS	–187 ± 40 (7)	**	NS
$\alpha 1(I&L) \beta 2(I232T&W237Q) \gamma 2L$	140 ± 50 (5)	NS	NS	–252 ± 52 (6)	**	NS
$\alpha 1(I&L) \beta 2 \gamma 2L(W252Q)$	145 ± 21 (5)	NS	NS	–469 ± 123 (6)	*	NS
$\alpha 1(I&L) \beta 2(TM1 Q237L) \gamma 2L$	116 ± 30 (3)	NS	NS	–396 ± 114 (15)	***	NS
$\alpha 1(I&L) \beta 2(CTVQ) \gamma 2L$	170 ± 30 (8)	**	NS	–1607 ± 314 (13)	NS	NS
$\alpha 1(I&L) \beta 2(TM1 W241L) \gamma 2L$	98 ± 21 (5)	NS	NS	–248 ± 72 (11)	***	NS
$\alpha 1(W245L) \beta 2 \gamma 2L$	63 ± 5 (5)	NS	—	–2019 ± 492 (7)	NS	—
$\alpha 1(W245L) \beta 2(TM1) \gamma 2L$	4 ± 1 (5)	NS	**	–2235 ± 342 (5)	NS	NS
$\alpha 1(W245L) \beta 2 \gamma 2(TM1)$	46 ± 10 (7)	NS	NS	–2426 ± 197 (7)	NS	NS

The first column presents the subunits transfected. The subsequent columns show data for activation by GABA: the mean EC₅₀ for fits of data with the Hill equation and the mean response to 1 mM GABA (at or close to the maximal response for all combinations). The results of statistical tests are given as in Table 1.

was significantly increased for receptors comprising $\alpha 1(I&L) + \beta 2 + \gamma 2L$ subunits (Table 3). Similarly, transferring the TM1 region to other subunits altered the EC₅₀ (Table 3), as did mutations in the transferred domains. The mean response elicited by 1 mM GABA was reduced for many of the subunit combinations, compared

with wild-type subunits (Table 3). This likely reflects a reduction in receptor assembly and transport, although some reduction in maximal probability of being open may occur (Akk *et al.* 2008).

We examined the correlations between pairs of values, to determine whether there were possible relationships

(e.g., a low maximal response might reflect a low maximal probability of being open, which might result in enhanced apparent potentiation). However, there was little correlation between pairs of values. The rank correlation coefficients were not significantly different from 0 for potentiation and EC_{50} , gating and EC_{50} , gating and potentiation, or gating and maximal response ($P > 0.12$ for each). The lack of correlation of potentiation with EC_{50} is consistent with the idea that the effects on activation by GABA and on potentiation by $3\alpha 5\alpha P$ are independent of each other.

The regression of rank potentiation on rank maximal response was weakly significant (slope = 0.6, $P = 0.03$), and of rank EC_{50} on rank maximal response (slope = -0.6, $P = 0.04$). That is, constructs that gave small maximal responses tended to have less potentiation and larger EC_{50} values. The correlation of potentiation with maximal response is of unknown significance, but the nature of the correlation is the opposite of that expected if, for example, small maximal responses resulted from low maximal probability of being open. In addition, we note that the data in Tables 1 and 3 show a group of constructs with both low maximal response and low potentiation. For constructs with maximal currents of 1.5 nA or more, potentiation ranged from none (potentiation ratio = 1.04, maximal response 1.8 nA) to maximal (4.98, 1.8 nA). The correlation of EC_{50} with maximal response is perhaps more robust, as there is a group of constructs with maximal response < 1 nA with EC_{50} values greater than 97 μM , and a second group with responses > 1 nA and EC_{50} values less than 64 μM . Some of the correlation might be explained by the possibility that our estimate of maximal response (response to 1 mM GABA) was actually submaximal for constructs with the largest EC_{50} values, but this seems unlikely to account for all of it. However, the significance of the correlation is unclear at present.

Discussion

These findings support the idea that the primary structure of the TM1 region is critical for steroid interaction with the GABA_A receptor. It was proposed that the steroid molecule also interacts with two residues in the TM4 region of the $\alpha 1$ subunit (Hosie *et al.* 2006). Both the $\beta 2$ and $\gamma 2L$ subunits have identical residues to $\alpha 1$ at the homologous positions, so this transfer does not address the question of additional binding regions.

It can be difficult to disentangle binding from transduction, particularly when the region of interest is close to the gating region of the receptor and the drug (steroid) is likely to affect interactions of the transmembrane helices. However, the observation that potentiation can be transferred among subunits of the GABA_A receptor provides circumstantial support to the idea that this domain of the receptor participates in steroid recognition (binding),

rather than being solely involved in transduction. If steroids bound elsewhere on the receptor, it seems less likely that the mechanism coupling the binding site to the transduction region would be normally operative when the transduction region was moved to a different subunit. Still, the fact that mutations in the TM1 region affect gating by GABA suggests that this region is involved not just in binding steroids but also in transduction of binding to potentiation. We also note that evolutionarily related subunits might diverge from a common ancestor in such a way that both α and non- α subunits contain binding sites for steroids, but the non- α subunits have lost the transduction mechanism. Distinguishing binding from transduction will require further work. It is clear that the interactions between steroid and receptor are relatively complicated and may involve an extended interface rather than a point-to-point, specific interaction (Akk *et al.* 2008; Li *et al.* 2009).

Previous work has indicated that steroid potentiation is mediated by an overall effect on receptor function, rather than a direct effect delimited by an action on a single subunit (Akk *et al.* 2009; Bracamontes & Steinbach, 2009; Bracamontes *et al.* 2011). That is, we have been able to produce receptors with wild-type or mutated TM1 regions, and wild-type or mutated GABA-binding sites in all combinations, and have found that the intact sites do not have to occur on the same α subunit to subserve potentiation. The present results extend this observation, by demonstrating that the TM1 region can be moved to subunits other than the α subunit and result in a receptor that shows steroid potentiation.

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Author contributions

Conception and design of the experiments: J.R.B., G.A. and J.H.S.; collection, analysis and interpretation of data: J.R.B., P.L., G.A. and J.H.S.; drafting the article or revising it critically for important intellectual content: J.R.B., P.L., G.A. and J.H.S. All authors approved the final version for publication.

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