

Insight into the Mechanism of Action of Neuroactive Steroids

Kendall D. W. Morris and Jahanshah Amin

Departments of Pharmacology & Therapeutics and Physiology & Biophysics, College of Medicine, University of South Florida

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ABSTRACT

Rho₁ receptor-channels (ρ_1 Rs) are GABA-gated chloride channels that exhibit slow kinetics, little desensitization, and inert pharmacology to most anesthetics, except for neuroactive steroids (NSs). NSs differentially modulate ρ_1 Rs dependent on the steric arrangement of the hydrogen atom at the fifth carbon position. In particular, the NS allotetrahydrodeoxycorticosterone (5 α -THDOC) potentiates, whereas 5 β -pregnane-3 α -ol-20-one (pregnanolone) and 5 β -dihydroprogesterone (5 β -DHP) inhibit ρ_1 GABA currents. Here, we used *Xenopus laevis* oocytes expressing ρ_1 Rs as a model system to study the mechanism of NS modulation. The second transmembrane residue, Ile307, was mutated to 16 amino acids. Subsequent testing of these mutants with 5 α - and 5 β -NSs, at equivalent GABA activity, showed the following paradigm. For 5 β -DHP, Ile307 mutation either altered the degree of inhibition or entirely reversed the direction of modulation, rendering 5 β -DHP a potentiator.

Dependent on the mutation, pregnanolone remained an inhibitor, transformed into a potentiator, or converted to inhibitor and potentiator based on concentration. The extent of mode reversal for both 5 β compounds showed a correlation with the side-chain hydrophilicity of the 307 residue. In contrast, Ile307 substitutions did not alter the direction of modulation for 5 α -THDOC but caused a significant increase in the level of potentiation. Paradoxical to their impact on the mode and/or the degree of modulation, none of the mutations altered the concentration range producing the response significantly for any of the above NSs. Moreover, preincubation of Ile307 mutants with 5 α or 5 β alone produced an equivalent effect on the activation time course. Based on the above data, a universal model is presented wherein anesthetic compounds like NSs can potentiate or inhibit the activity of ligand-gated ion channels distinct from interaction with alternative binding sites.

Metabolites of sex and stress hormones, known as neuroactive steroids (NSs), represent a large class of endogenous compounds within the central nervous system (CNS) (Covey et al., 2001; Lambert et al., 2001; Stoffel-Wagner, 2001; Mellon and Griffin, 2002). In vivo, NSs seem to play an important role in the establishment of mood and behavior states. In the clinical setting, administration of NSs can produce a dose-dependent graded effect upon the function of the CNS that ranges from sedation to induction of anesthesia (Purdy et al., 1991; Gray et al., 1992; Frye and Duncan, 1994; Romeo et al., 1998; Girdler et al., 2001; Mellon and Griffin, 2002). NSs mediate their effect by targeting membrane-embedded proteins known as ligand-gated ion channels and thereby alter synaptic transmission within the CNS. One of the most diversified and ubiquitously expressed classes of inhibitory ligand-gated ion channels is the GABA_A receptor (GABA_AR). NSs are modulators and can be direct agonists of GABA_ARs. In this regard, their action closely resembles that of intravenous anesthetic barbiturates (Harrison and Simmonds, 1984;

Majewska et al., 1986; Barker et al., 1987; Peters et al., 1988; Turner et al., 1989; Morrow et al., 1990; Puia et al., 1990; Majewska, 1992; Twyman and MacDonald, 1992; Woodward et al., 1992; Zaman et al., 1992; Kokate et al., 1994; Shen et al., 2000).

The GABA_AR is a pentameric protein complex composed of closely related subunits. Each subunit is postulated to cross the membrane bilayer four times, where the second transmembrane domain is the ion channel (Olsen, 1998; Sieghart, 2000). Closely related to GABA_A are GABA_C ρ_1 receptor-channels (ρ_1 Rs). Compared with GABA_ARs, ρ_1 Rs are insensitive to most anesthetics except in the case of NSs (Shimada et al., 1992; Morris et al., 1999; Chebib and Johnston, 2000; Qian and Ripps, 2001). Chimera studies between the subunits of GABA_ARs and ρ_1 Rs and mutagenesis have been essential to mapping the domain and pinpointing the amino acids important for a number of anesthetics. These studies demonstrate that ρ_1 Ile307 and Trp328, positioned within the second and third transmembrane domains (TM2 and TM3, respectively), play an important role in anesthetic action (Amin, 1999; Belelli et al., 1999; Walters et al., 2000). Mutation of ρ_1 Trp328 to Ala or Met (or any hydrophobic residues),

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ABBREVIATIONS: NS, neuroactive steroid; CNS, central nervous system; GABA_AR, GABA_A receptor; ρ_1 R, ρ_1 receptor-channel; TM, transmembrane domains; OR2, oocyte Ringer's 2; DHP, dihydroprogesterone; 5 α -THDOC, allotetrahydrodeoxycorticosterone; pregnanolone, 5 β -pregnane-3 α -ol-20-one; HI, hydrophobicity index.

found at the equivalent TM3 position within the α_1 and β_2 subunits of the GABA_A receptor, respectively, confers barbiturate sensitivity to ρ_1 Rs (Amin, 1999). The residue equivalent to ρ_1 Ile307 within the α_1 subunit is serine. Mutation of ρ_1 Ile307 to Ser also confers barbiturate sensitivity to ρ_1 Rs (Belelli et al., 1999). Moreover, mutations of both Ile307 and Trp328 to their amino acid counterpart within the α_1 and β_2 subunit impart benzodiazepine sensitivity to ρ_1 Rs (Walters et al., 2000).

NSs differentially modulate the ρ_1 R dependent on the steric arrangement of the hydrogen atom at the fifth carbon position of the cholesterol backbone (Morris et al., 1999). The 5 α position of the hydrogen atom significantly reduces the bend within the cholesterol backbone in comparison to 5 β position rendering the 5 α -NSs (e.g., 5 α -THDOC) potentiators, and 5 β -NSs (e.g., 5 β -DHP and pregnanolone) inhibitors of the ρ_1 R GABA current. It is interesting that the position of the hydrogen atom at the 5th carbon does not have an effect on the mode of modulation of the NS upon the GABA_AR because both 5 α - and 5 β -NSs are positive modulators of GABA_ARs.

We used the ρ_1 R as a model system to study the molecular mechanism of NS action. The ρ_1 R offers a number of advantages over GABA_AR systems including slow kinetics of activation and deactivation, low rate of desensitization (Amin and Weiss, 1994), and 5-fold degeneracy (comparison with GABA_AR composed of closely related but not identical subunits). The effect of mutation at position 307 (and others) within the TM2 on the modulation by 5 α -THDOC, pregnanolone, and 5 β -DHP was determined. The Ile307 mutations impacted the mode and the degree of modulation for 5 β compounds, either by entirely reversing the direction of modulation from inhibition to potentiation for both 5 β -NS or by producing biphasic activity for pregnanolone. In contrast to 5 β compounds, the effect of these mutations on 5 α -THDOC was to significantly increase the level of potentiation without altering the mode of modulation. However, the marked impact of Ile307 mutations on direction of the NS action did not parallel a significant alteration in the NS concentration range producing the response. Furthermore, preincubation with 5 α or 5 β alone produced an equivalent effect on the NS activation time course. Based on these data, we have proposed a unique model for NS-dependent modulation of ligand-gated ion channels.

Materials and Methods

Single amino acid substitutions were created using GeneEditor in vitro site-directed mutagenesis system (Promega, Madison, WI) within the pSp72 plasmid vector (Promega). The restriction enzyme SspI was used to linearize the plasmid vector containing the mutated or the wild-type human ρ_1 cDNA. Linearization with this restriction enzyme leaves a tail on the cRNA of a few hundred base pairs, which may enhance the stability of the cRNA within the oocyte. The cRNA was synthesized from this template using the T7 Megascript in vitro transcription kit (Ambion, Austin, TX). The cRNA was run on a 1% agarose gel containing formaldehyde to determine its quality. The optical density of the cRNA was also determined to assess the quantity of the cRNA.

The *Xenopus laevis* frogs were anesthetized using 0.1% Tricaine before ovariectomy. After surgery, the oocytes were placed in a calcium-free oocyte Ringer's solution (OR2; 83.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2 mM sodium pyruvate, 1 mM Na₂HPO₄, and 5

mM HEPES, pH 7.5). Stage V and VI oocytes were isolated and maintained in OR3 (82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 2 mM sodium pyruvate, 1 mM Na₂HPO₄, 5 mM HEPES, 50 U/ml penicillin, and 50 mg/ml streptomycin, pH 7.5) plus 2% horse serum at 18°C.

The micropipettes for cRNA injection were pulled on a Sutter P87 puller (Sutter Instrument Co., Novato, CA). The tips were cut using microscissors under a dissecting microscope. The cRNA was drawn into the micropipette using negative pressure and then injected into the oocytes with positive pressure from a Picospritzer II device (General Valve Co., Fairfield, NJ).

Two to three days after injection, the oocytes were placed in a recording chamber with a volume of approximately 50 μ l. This chamber has an inlet at the top and outlet at the bottom allowing continuous gravity-fed perfusion of the oocytes with either control or test solution. Twenty glass reservoirs are connected to four six-way valves. Each of these valves is also connected to a 2-liter container with OR4 (92.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 5 mM HEPES, pH 7.5). These six-way valves are connected to one four-way valve. In this setup, 20 different test solutions could be applied to the chamber at a given time.

The NSs were purchased from Steraloids (Newport, RI), and stock solutions of 10 mM were made in dimethyl sulfoxide. On the day of recording, test solutions were made by adding these stock solutions to rapidly stirring recording OR2. The hydrophobic nature of these compounds precluded the creation of a concentration greater than 20 μ M. The highest concentration of the vehicle solution (0.2%) did not alter the GABA-induced currents from either the wild-type or the mutated ρ_1 R.

Recording electrodes were pulled on a Narishige pp-83 vertical puller (Narishige Scientific Instrument Lab, Tokyo, Japan). The electrodes were filled with 3 M KCl. Oocytes were clamped at -70 mV using a Turbo TEC-05 npi (Adams and List, Westbury, NY). The output was recorded on a Gould TA 240 chart recorder and digitized using Pulse 8.3 (HEKA Elektronik, Lambrecht, Germany).

For all mutated ρ_1 Rs, the concentration-response relationships with GABA were constructed to determine the effective concentration producing 50% (EC₅₀) and 9% of the maximal current (EC₉). The calculated EC₉ obtained from the concentration-response relationship data were tested by comparing the GABA-induced current at EC₉ with the maximal current evoked by a GABA concentration 50 times the EC₅₀ value. If needed, the EC₉ was adjusted to a concentration that empirically produced approximately 9% of the maximal response. The standard deviation for EC₉ measurements is within 4%, which could result from the level of expression or batch variation within the oocytes. Overall, this apparent variation in EC₉ has a greater effect on the level of potentiation than inhibition. For example, a decrease from EC₁₄ to EC₄ GABA resulted in an increase in the level of potentiation by 5 α -THDOC (10 μ M) from $95 \pm 7\%$ to $210 \pm 16\%$ for wild-type ρ_1 R (Morris et al., 1999). On the other hand, the level of inhibition is affected less by deviation in EC calculation. For wild-type ρ_1 R, the level of inhibitions by 5 β -DHP (10 μ M) were $-30 \pm 4\%$ and $-26 \pm 2\%$ in the presence of EC₄ and EC₁₄ GABA, respectively (Morris et al., 1999).

For all the NS experiments, the control GABA solution was applied a minimum of two times before the NS application, to assure that the control GABA currents were consistent in amplitude. The time of application for GABA and GABA plus NSs was limited to 3 min to standardize these experiments.

Percentage potentiation and inhibition was calculated using the equation PP or $PI = 100 \times (I_{NS} - I_{GABA})/I_{GABA}$, where I_{NS} is the current amplitude in the presence of NS and GABA, and I_{GABA} is the control current amplitude in the presence of EC₉ GABA. In this setting, a 100% potentiation would represent a current twice the amplitude of the control current.

Two or more experiments were required to determine the quantitative measurements for the NSs for each mutant, using three or more oocytes per mutant. Determinations of statistical significance

were carried out using either the student *t* test or ANOVA, as appropriate, requiring a *p* value of less than 0.05. All data are presented as mean \pm S.E.M.

Results

The Mutation of ρ_1 Ile307 to Ser Reversed the Direction of 5β -DHP modulation. The residue equivalent to the ρ_1 Ile307 within the α subunit of GABA_AR is Ser. Previous studies have shown that the mutation of Ile307 to Ser in the ρ_1 R can impart pentobarbital and benzodiazepine sensitivity to ρ_1 Rs (Amin, 1999; Belelli et al., 1999; Walters et al., 2000). We investigated the effect of the Ile307-to-Ser substitution (ρ_{1307S}) on modulation by 5β -DHP. The cRNA corresponding to ρ_{1307S} was injected into oocytes; 3 days after injection, the GABA concentration response relationship for the ρ_{1307S} receptor-channel ($\rho_{1307S}R$) was established. From these experiments, the effective GABA concentration producing 9% of the maximal current (EC_9) was determined (see *Materials and Methods*). The EC_9 was chosen because 1) the activation for ρ_1 R is slow at such GABA concentrations, facilitating the extraction of the details of the activation kinetics (see below), and 2) the action of the NSs are particularly exaggerated at such submaximal GABA concentrations (see *Materials and Methods*). The 5β -DHP modulation of $\rho_{1307S}R$ in the presence of EC_9 GABA was determined next. Figure 1 shows representative current traces of GABA, GABA plus 1, 5, 10, or 20 μ M 5β -DHP, and the 5β -DHP concentration-response relationship for wild-type (gray line) and $\rho_{1307S}R$ (green line). The control GABA current traces for wild-type and mutant receptor-channels were scaled to the same amplitude to discern the difference between the 5β -DHP actions on the wild-type versus mutant. Single TM2 mutation of Ile307 to Ser reversed the direction of 5β -DHP modulation from inhibition to potentiation. One, 5, 10, and 20 μ M 5β -DHP inhibited the wild-type GABA (EC_9) currents by -5 ± 2 , -27 ± 5 , -39 ± 4 , and $-46 \pm 4\%$, respectively (see also Table 2). In contrast, the GABA-evoked currents for $\rho_{1307S}R$ were increased by 9 ± 6 , 34 ± 7 , 75 ± 28 , and $163 \pm 34\%$ at the same concentrations of 5β -DHP, respectively. The EC_{50} value for the 5β -DHP positive modulation of $\rho_{1307S}R$ could not be predicted because even at the highest feasible concentration (20 μ M; see *Materials and Methods*), the maximal current could neither be reached nor extrapolated. However, both inhibition and potentiation by 5β -DHP on ρ_1 and $\rho_{1307S}R$ was initiated at 5 μ M.

The Hydrophobicity of Residue 307 Determines the Polarity of Modulation by 5β -DHP. To investigate the role of residue 307 in 5β -DHP-dependent modulation of ρ_1 Rs, the ρ_1 Ile307 was mutated to Val, Cys, Met, Phe, Ala, Thr, Gly, Pro, Ser, Trp, Asn, His, Gln, Glu, Lys, and Arg residues. The side chains of these residues represent a complete spectrum in size, charge, and hydrophobicity. These mutations were created to determine 1) the amino acid side-chain properties at position 307 that are important for conserving the inhibitory action of 5β -DHP, 2) the key amino acid side-chain requirements in reversing the direction of 5β -DHP's activity from an inhibitor to a positive modulator, and 3) whether any amino acid substitution will shift the 5β -DHP concentration-response range (1 to 20 μ M) significantly (i.e., to produce a change within the concentration response curve

such that maximal potentiation is reached or no response is seen across the concentrations tested)?

For all mutants, GABA concentration-response relationships were constructed to determine first the viability of these mutated receptor-channels; second, the shift in GABA sensitivity; and finally, the GABA EC_9 value for each mutant to test 5β -DHP action at equivalent receptor-channel activity. From the 16 amino acid substitutions, Pro did not yield GABA-gated activity (up to 10 mM), Lys and Phe produced a low maximal, and Phe resulted in receptor-channels that had constitutive activity (Table 1). With the above exceptions, all substitutions at position 307 produced receptor-channels that exhibited similar or higher sensitivity to GABA compared with wild-type (Table 1). The size or the charge of the amino acid side chain at position 307 had little effect on

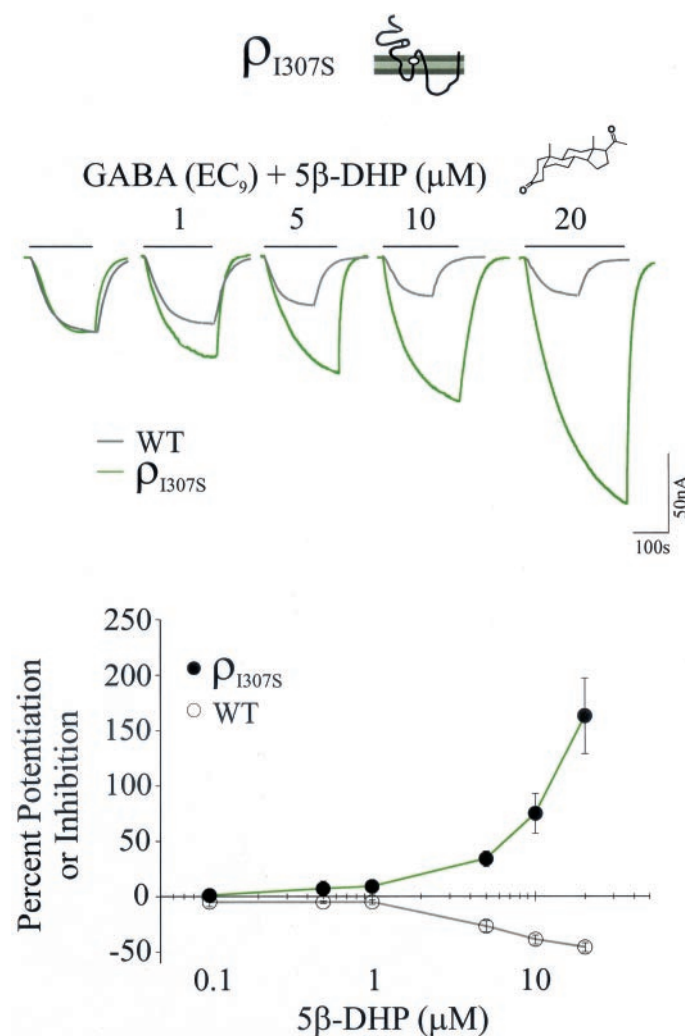


Fig. 1. The ρ_1 Ile307 mutation to Ser reverses the direction of 5β -DHP's modulation from inhibition to potentiation. Top, representative current traces of GABA (EC_9) and GABA plus 1, 5, 10, or 20 μ M 5β -DHP for wild-type (gray line) and $\rho_{1307S}R$ (green line). The control GABA current traces for wild-type and $\rho_{1307S}R$ are scaled to the same amplitude to discern the relative magnitude of 5β -DHP action on wild-type versus mutant. Scale bar representing the current magnitude only applies to mutant current traces. Thick lines above each current trace represent the duration of GABA or GABA plus NS application for ρ_{1307S} traces only. Bottom, 5β -DHP concentration-response relationship. Ile307-to-Ser substitution reversed the action of 5β -DHP from an antagonist to a positive modulator. Note that the opposing effect of 5β -DHP on ρ_1 R and $\rho_{1307S}R$ was produced within the same concentration range.

potency. For example, Gly and Trp substitutions (opposite in terms of size) resulted in receptor-channels with GABA EC_{50} of 0.36 ± 0.019 and $0.53 \pm 0.027 \mu\text{M}$, whereas for Arg and Glu substitution (opposite in terms of charge), these values were 1.21 ± 0.04 and $2.71 \pm 0.45 \mu\text{M}$, respectively. Overall, the EC_{50} for the 307 mutants varied from 0.09 (for ρ_{1307H} , $EC_{50} = 0.09 \pm 0.003$) to $2.71 \mu\text{M}$ (ρ_{1307E} , $EC_{50} = 2.71 \mu\text{M} \pm 0.45$). The mutational tolerance in terms of the GABA potency indicates that position 307 may not be a key component of the GABA-binding domain. However, the maximal GABA-induced current for these mutants ranged from approximately 10% to 100% of the wild type, suggesting that residue 307 may play a role in the assembly, transduction, and/or gating processes of the GABA-dependent activation within the ρ_1R .

The modulatory effect of 5 β -DHP was determined for all 13 Ile307 mutants responsive to GABA (Val, Cys, Met, Ala, Thr, Gly, Ser, Trp, Asn, His, Gln, Glu, and Arg substitution). Figure 2 shows bar graphs representing the 5 β -DHP action at 1, 5, 10, and 20 μM for the wild-type and Ile307 mutants (see also Table 2) in the presence of their respective EC_9 GABA concentrations (the amplitudes of GABA-elicited currents at EC_9 ranged between approximately 20 and 200 nA). The rainbow-like color spectrum in Fig. 2 corresponds to the hydrophobicity index (HI) of the substituted amino acid based on the calculation by Kyte and Doolittle (1982). The color spectrum ranges from violet, symbolizing the most hydrophobic (Ile, HI = 4.5), to red, representing the most hydrophilic residue (His, HI = -4.5). The bar graph represents the degree of inhibition/potentiality by 5 β -DHP for the mutant constructs and is arranged by polarity and the relative level of 5 β -DHP activity, from the mutant that causes the highest inhibition on the far left to those producing the most

potentiation on the far right. Examination of the color spectrum created by these data demonstrated a correlation between the hydrophilicity of the side chain and the direction (from inhibitory to potentiating) of 5 β -DHP modulation. The highest level of 5 β -DHP inhibitions occurred with the most hydrophobic amino acids, and a decline in inhibitory activity became evident as the hydrophilicity of the substituted residue increased (see also Table 2). For example, 5 β -DHP (20 μM) inhibited the ρ_1 (Ile, HI = 4.5) and ρ_{1307V} (Val, HI = 4.2) GABA currents by -46 ± 4 and $-40 \pm 6\%$, respectively. In comparison, 20 μM 5 β -DHP displayed little inhibition for ρ_{1307M} ($-9 \pm 14\%$; HI = 1.9) or ρ_{1307T} ($-14 \pm 5\%$; HI = 0.7). Finally, with hydrophilic substitutions such as Ser (HI = -0.4), Arg (HI = -4.5), Glu (HI = -3.5), and Gln (HI = -3.5), 5 β -DHP was transformed into a potentiator. In the presence of 20 μM 5 β -DHP, the GABA current for ρ_{1307S} , ρ_{1307R} , ρ_{1307E} , and ρ_{1307Q} Rs increased by 163 ± 34 , 127 ± 8 , 213 ± 23 , and $254 \pm 28\%$, respectively. The change in the action of 5 β -DHP was independent of the size (see Gly and Trp substitutions) or the charge (see Arg versus Glu) characteristics of the substituted residue. However, the change in color spectrum versus activity indicates that the hydrophilicity of the amino acid at position 307 determines whether 5 β -DHP acts as a negative or positive modulator.

Independent of potentiation or inhibition, modulation of Ile307 mutants by 5 β -DHP first became evident at 5 μM . Therefore, the marked impact on the polarity of the 5 β -DHP's modulation paralleled little or no change in the lowest concentration required to produce a response. Moreover, none of Ile307 mutations altered the concentration response curve such that potentiation was maximal for a number of concentration (shift of concentration response to the left) or no response was seen across the concentrations tested (shift of concentration response to the right).

Biphasic Modulation of the $\rho_{1307S}R$ by Pregnanolone.

The sole structural difference between 5 β -DHP and its metabolite, pregnanolone, is a hydroxyl group versus a double-bound oxygen on the third carbon. Although both 5 β -DHP and pregnanolone inhibited wild-type ρ_1 Rs, there were differences between their actions on the wild-type receptor-channel. First, pregnanolone showed a higher potency than 5 β -DHP (pregnanolone $IC_{50} = 0.6 \pm 0.09 \mu\text{M}$; 5 β -DHP $IC_{50} = 3.43 \pm 0.30 \mu\text{M}$). Second, the maximum inhibition for pregnanolone occurred at 10 μM (-46 ± 5), whereas at 20 μM , the inhibitory action was reduced (-34 ± 5). By comparison, 5 β -DHP produced a progressively greater inhibition with maximal inhibition at 20 μM (see Figs. 1 and 3).

To examine whether ρ_1 Ile307 mutation to serine would also produce a reversal in the modulation of another 5 β compound, we determined the pregnanolone concentration-response relationship for $\rho_{1307S}R$ (at 0.1, 0.5, 1, 5, 10, and 20 μM) in the presence of the EC_9 GABA. Figure 3 shows the pregnanolone concentration-response relationship (and the current traces thereof) for wild-type (gray lines) and $\rho_{1307S}R$ (red lines). The control GABA current traces (EC_9) are shown at the same amplitude to facilitate comparison between pregnanolone-dependent modulation of the wild type and $\rho_{1307S}R$. Pregnanolone inhibited $\rho_{1307S}R$ GABA currents between 0.1 and 5 μM (see Table 3); the maximal apparent inhibition occurred at 1 μM ($-56 \pm 4\%$). The pregnanolone inhibitory trend showed a reversal at 5 μM ($-43 \pm 2\%$) and nearly vanished at 10 μM ($-9 \pm 6\%$). In contrast, at 20 μM , preg-

TABLE 1

The EC_{50} and the Hill coefficient values of mutated ρ_1R

The EC_{50} and Hill coefficient were determined by fitting the concentration-response relationship to the following logistic equation using Sigma Plot 4.0: $I = I_{\max}/[1 + (EC_{50}/[C])^{n_H}]$, where I is the peak current at a given concentration of GABA $[C]$, I_{\max} is the maximal current, EC_{50} is the concentration of GABA producing 50% of maximal inhibition, and n_H is the Hill number. All data are presented as mean \pm S.E.M.

Receptor-Channel	EC_{50}	Hill	Range of I_{\max}^a nA	n
ρ_1	1.00 ± 0.15	2.02 ± 0.41	100–4000	21
ρ_{1307S}	0.25 ± 0.011	3.32 ± 0.09	265–750	6
ρ_{1307A}	0.22 ± 0.024	1.76 ± 0.13	608–1100	4
ρ_{1307T}	0.32 ± 0.028	2.46 ± 0.12	117–455	4
ρ_{1307N}	0.31 ± 0.014	2.55 ± 0.09	670–1115	6
ρ_{1307C}	0.15 ± 0.009	3.00 ± 0.14	475–830	4
ρ_{1307Q}	0.22 ± 0.018	2.59 ± 0.26	292–1385	3
ρ_{1307G}	0.36 ± 0.019	2.16 ± 0.19	792–1050	3
ρ_{1307M}	0.26 ± 0.019	2.42 ± 0.12	720–1205	4
ρ_{1307H}	0.09 ± 0.003	2.47 ± 0.15	233–800	4
ρ_{1307W}	0.53 ± 0.027	2.69 ± 0.10	530–557	3
ρ_{1307E}	2.71 ± 0.45	0.92 ± 0.14	600–1550	3
ρ_{1307V}	0.19 ± 0.011	2.54 ± 0.15	203–670	4
ρ_{1307R}	1.21 ± 0.04	1.98 ± 0.10	280–660	4
ρ_{1307K}	^b		140–215	
ρ_{1307F}	^{b,c}		70–198	
ρ_{1307P}	^d			
ρ_{1306S}	^d			
ρ_{R287A}	0.39 ± 0.017	3.39 ± 0.19	400–602	3
ρ_{P294A}	2.54 ± 0.17	2.07 ± 0.07	610–1900	7

^a Low maximum current

^b Spontaneous current

^c No current detected up to 1 mM GABA

^d Range of I_{\max} from the concentration response relationship experiments

nanolone became a positive modulator of ρ_{I307S} , increasing the GABA currents by $173 \pm 20\%$. Thus, pregnanolone exhibited biphasic activity, producing inhibition at lower concentrations and potentiation at the highest concentration. Although the Ile307-to-Ser mutation produced a marked transformation in pregnanolone mode and degree of modulation, the apparent pregnanolone sensitivity for $\rho_{I307S}R$ was not altered. For both mutant and wild-type receptor-channels, pregnanolone initiated a response at approximately $0.5 \mu M$.

Mono- and Biphasic Modulation of Ile307 Mutants by Pregnanolone. Pregnanolone inhibited or potentiated the $\rho_{I307S}R$ GABA currents, dependent on the concentration. To determine the side-chain properties of residue 307 responsible for this dual action, pregnanolone concentration-response relationships were established for each of the above 13 ρ_1R mutants. Figure 4 depicts a bar graph representing pregnanolone modulation (at 0.1, 0.5, 1, 5, 10, and $20 \mu M$ concentrations) of 13 mutants and the wild type in the presence of their respective EC_9 GABA (see also Table 3). The preg-

nanolone data are presented using the same color-coded hydrophathy index as with 5β -DHP experiments. The color spectrum progresses from violet, for the most hydrophobic, to red, representing the most hydrophilic amino acids. Given the bimodal nature of pregnanolone modulation, the data are arranged from left to right based on the number of pregnanolone concentrations producing inhibition for a given mutant. For example, the data for ρ_{I307G} is placed to the left of ρ_{I307E} , because four concentrations (0.1, 0.5, 1, and $5 \mu M$) of pregnanolone inhibited ρ_{I307G} , whereas only three (0.1, 0.5, and $1 \mu M$) inhibited the $\rho_{I307E}R$ GABA current. For those mutations that were inhibited by the same number of concentrations, the percentage potentiation at $20 \mu M$ pregnanolone was used to assign ranking, from least (left) to greatest potentiation (right). For example, the data for ρ_{I307W} is placed to the right of ρ_{I307N} because, at $20 \mu M$ pregnanolone, the potentiation was greater for $\rho_{I307W}R$ than for $\rho_{I307N}R$. Similar to 5β -DHP, the color (hydrophilicity) change versus pregnanolone modulation profile of the mutants

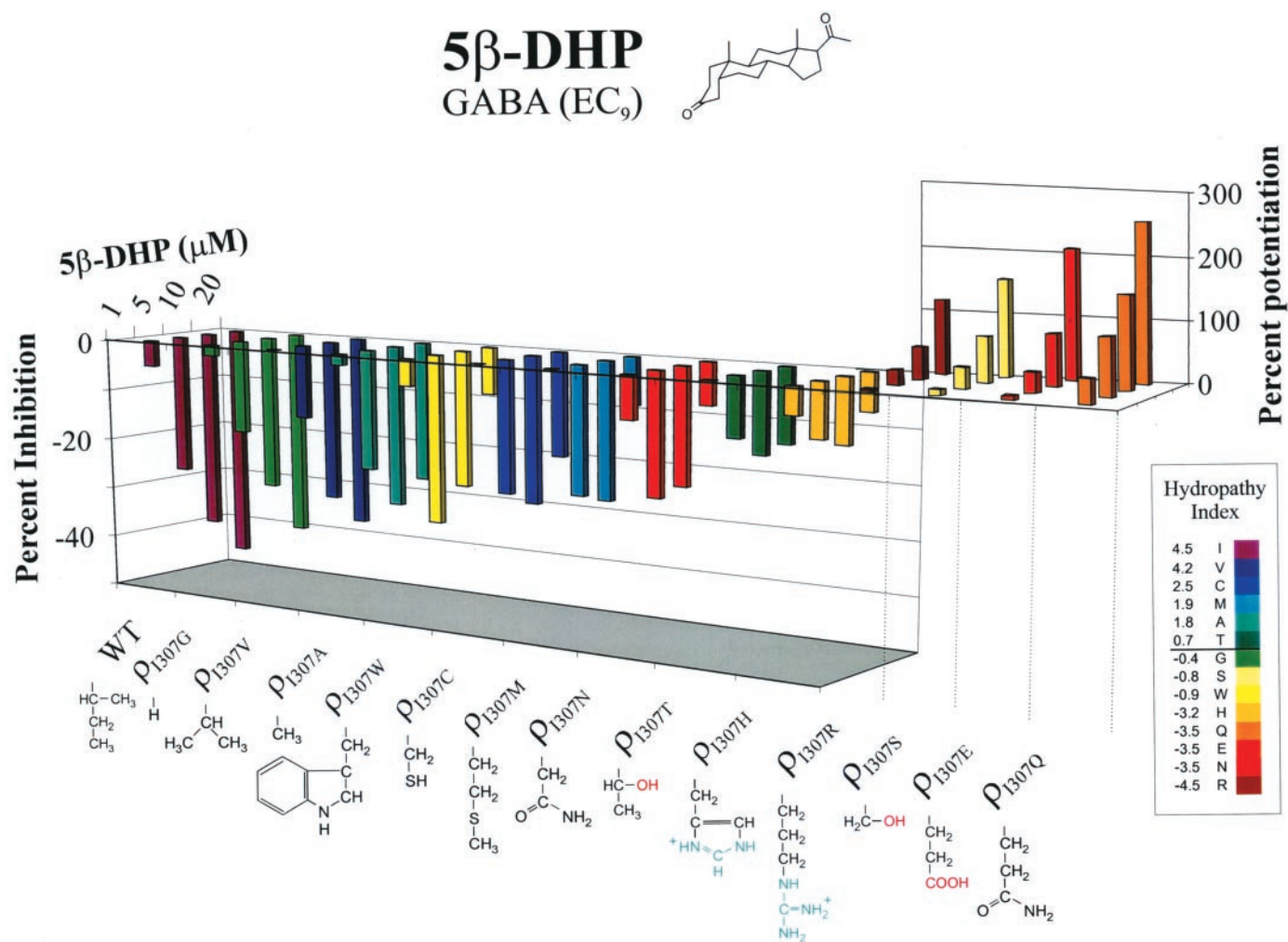


Fig. 2. The relationship between polarity of 5β -DHP's modulation and hydrophobicity of the amino acid side chain at position 307. The modulatory effect of 5β -DHP at 1, 5, 10, and $20 \mu M$ for all thirteen Ile307 mutants and wild-type is shown. The rainbow-like color spectrum is a depiction of the hydrophobicity index of the substituted amino acid based on the calculation by Kyte and Doolittle (1982). The color code (right) depicts the hydrophobicity index and ranges from violet, symbolizing the most hydrophobic, to red, representing the most hydrophilic residue. The bar graph is the degree of inhibition/potentiation by 5β -DHP for the mutant constructs and are arranged (from left to right) by the relative level and the polarity of 5β -DHP activity, with mutants causing the most inhibition positioned on the far left to those producing the highest potentiation on the far right (see Table 2 for S.E.M.). Hydrophobicity of the amino acid at position 307 seemed to be the essential determinant of the polarity of 5β -DHP's modulation. Structures of the amino acid side chains are also depicted to facilitate examination of the data with respect to the size or charge characteristics of the mutated residue.

showed a correlation between the hydrophilicity of the side chain and the direction of modulation for pregnanolone. Pregnanolone was predominately inhibitory for Ile (wild type, HI = 4.5), Val (HI = 4.2), and Cys (HI = 2.5), the three most hydrophobic residues. With a decrease in hydrophobicity of the substituted residue, pregnanolone showed potentiation starting with the highest concentration (20 μ M). For example, for Thr (HI = 0.7) and Ser (HI = -0.4) substitutions, 10 μ M pregnanolone decreased the GABA currents by -33 ± 6 and $-9 \pm 6\%$, whereas 20 μ M increased the GABA currents by 70 ± 6 and $173 \pm 20\%$, respectively. The inhibitory action of pregnanolone was further reduced with Glu, Asn, Trp, and Arg residues. Finally, pregnanolone completely transformed into a potentiator across the concentration range for Gln substitution (HI = -3.5). At 0.1, 0.5, 1, 5, 10, and 20 μ M concentration, pregnanolone potentiated the ρ_{I307Q} GABA currents by 3 ± 1 , 13 ± 5 , 23 ± 8 , 125 ± 22 , 249 ± 31 , and $531 \pm 66\%$, respectively. It is interesting to note that pregnanolone is a positive modulator of GABA_A receptors, where the equivalent amino acids to the Ile307 within the α , β , and γ subunits are Ser and Asn; these residues are significantly more hydrophilic than Ile. The pregnanolone sensitivity, however, was not altered significantly by any of Ile 307 substitution. Thus, paradoxical to their impact on the mode of modulation, none of the mutations greatly changed the lowest pregnanolone concentration required to initiate a response.

We mutated Ile306, Pro294, and Arg287 within the TM2 of the ρ_1 R to assess the specificity of position 307 within the TM2 in conferring the biphasic modulation to pregnanolone. The mutation of Ile at position 306 to a serine did not yield a functional receptor-channel. However, the expression of ρ_{P294A} and ρ_{R287A} cRNA produced receptor-channels with GABA EC₅₀ values of 0.39 ± 0.017 and 2.54 ± 0.19 , respectively (Table 1). The pregnanolone concentration-response relationships for both ρ_{P294A} and ρ_{R287A} were determined at EC₉ GABA. Similar to the wild type, pregnanolone inhibited the GABA currents for both ρ_{P294A} R and ρ_{R287A} R across the entire concentration range (Table 3). Thus, among these residues, mutation of position 307 seems to be specific in imparting biphasic modulation to pregnanolone.

In summary, depending on the Ile307 mutation, pregnanolone remained an inhibitor, transformed into a potentiator, or converted to inhibitor and potentiator of GABA cur-

rents based on concentration. The direction of pregnanolone modulation seemed dependent on the hydrophilicity of residue 307. Finally, a lack of mutational effect of other TM2 residues on pregnanolone modulation attested to the notion that the residue 307 may be uniquely positioned in controlling the mode of modulation.

Mutation of Ile307 Alters the Degree but Not the Mode of Modulation by 5 α -THDOC. Within 5 α -THDOC, the 5 α positioning compared with the 5 β positioning of the hydrogen atom reduces the bend within the cholesterol backbone and thereby increases the overall length. Previous studies have shown that 5 α -NSs including 5 α -THDOC are positive modulators of wild-type ρ_1 Rs (Morris et al., 1999). We

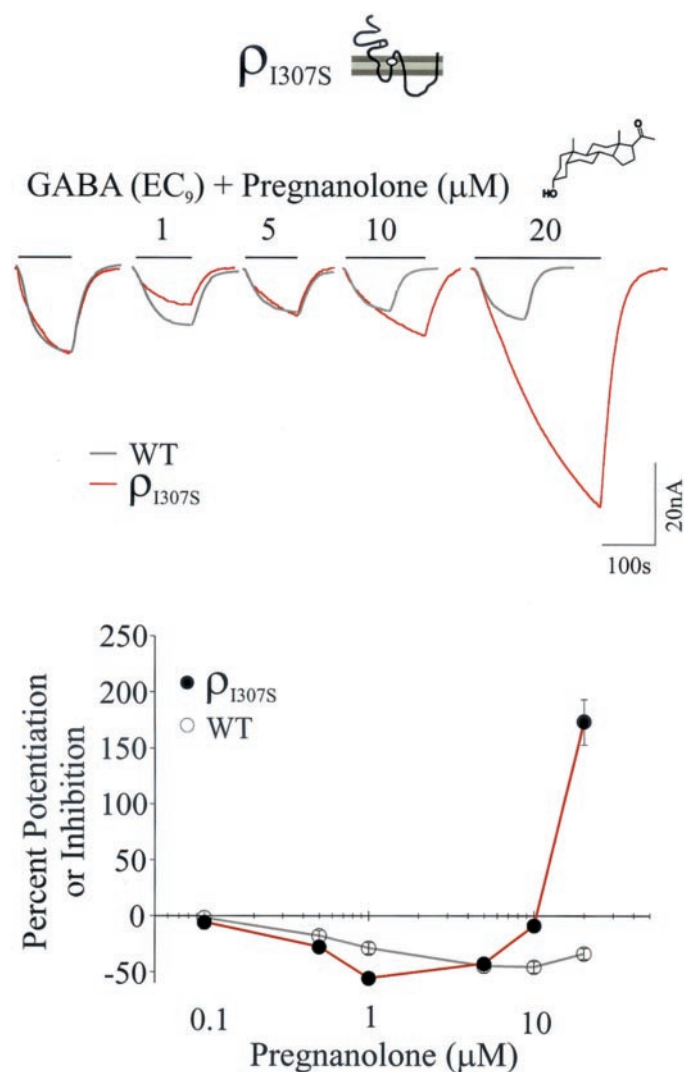


Fig. 3. Pregnanolone inhibits and potentiates the GABA currents of ρ_{I307S} R dependent on concentration. Top, current trace; bottom, concentration-response relationship for GABA (EC₉) and 0.1, 0.5, 1, 5, 10, or 20 μ M pregnanolone for wild-type (gray lines) and ρ_{I307S} R (red lines). The control GABA current traces (for both wild-type and mutant) are shown at the same amplitude to allow a direct comparison between pregnanolone-dependent modulation of the wild-type and ρ_{I307S} R. The scale bar representing the current magnitude applies only to mutant current traces. Thick lines above each current trace represent the duration of GABA or GABA plus NS application for ρ_{I307S} traces only. Pregnanolone inhibited the ρ_{I307S} R's GABA currents between 0.1 and 5 μ M concentrations but had little to no effect at 10 μ M. In contrast, 20 μ M pregnanolone significantly potentiated the ρ_{I307S} R GABA current.

TABLE 2

The 5 β -DHP-dependent modulation of Ile307 mutants in the presence their respective EC₉ GABA

All data are presented as mean \pm S.E.M.

Subunit	1 μ M	5 μ M	10 μ M	20 μ M	n
ρ_{I307}^*	-5 ± 2	-27 ± 5	-39 ± 4	-46 ± 4	6
I_{307G}	-2 ± 4	-18 ± 3	-30 ± 8	-40 ± 6	5
I_{307V}	1 ± 4	-14 ± 4	-31 ± 5	-37 ± 5	4
I_{307A}	-2 ± 6	-23 ± 5	-31 ± 10	-27 ± 10	3
I_{307W}	-5 ± 4	-32 ± 9	-26 ± 4	-9 ± 3	3
I_{307C}	1 ± 2	-25 ± 2	-28 ± 3	-20 ± 6	4
I_{307M}	0 ± 3	-24 ± 5	-26 ± 7	-9 ± 14	3
I_{307N}	-8 ± 4	-23 ± 4	-22 ± 4	-8 ± 7	4
I_{307T}	2 ± 7	-11 ± 6	-15 ± 4	-14 ± 5	3
I_{307H}	-5 ± 0.2	-10 ± 5	-12 ± 4	-7 ± 3	3
I_{307R}	4 ± 1	25 ± 4	54 ± 7	127 ± 8	4
I_{307S}	9 ± 6	34 ± 7	75 ± 18	163 ± 34	6
I_{307E}	6 ± 6	32 ± 15	83 ± 19	213 ± 23	4
I_{307Q}	37 ± 12	89 ± 23	145 ± 21	254 ± 28	3

TABLE 3
Pregnanolone-dependent modulation of Ile307 mutants in the presence their respective EC₉ GABA
All data are presented as mean ± S.E.M.

Subunit	0.1 μM	0.5 μM	1 μM	5 μM	10 μM	20 μM	n
ρ1*	-2 ± 2	-18 ± 5	-29 ± 5	-45 ± 6	-46 ± 5	-34 ± 5	4
I ₃₀₇ V	-1 ± 3	-31 ± 7	-37 ± 5	-45 ± 2	-32 ± 1	-16 ± 1	3
I ₃₀₇ C	-11 ± 4	-47 ± 6	-67 ± 4	-64 ± 4	-41 ± 2	16 ± 3	4
I ₃₀₇ T	-19 ± 7	-54 ± 5	-63 ± 5	-63 ± 3	-33 ± 6	70 ± 6	5
I ₃₀₇ S	-6 ± 6	-28 ± 3	-56 ± 4	-43 ± 2	-9 ± 6	173 ± 20	3
I ₃₀₇ A	-27 ± 2	-54 ± 4	-53 ± 3	-45 ± 4	14 ± 12	172 ± 36	4
I ₃₀₇ G	-12 ± 9	-54 ± 4	-63 ± 6	-49 ± 6	63 ± 20	206 ± 41	4
I ₃₀₇ E	-7 ± 2	-18 ± 2	-25 ± 3	1 ± 3	66 ± 14	151 ± 18	6
I ₃₀₇ M	-7 ± 2	-4 ± 6	-2 ± 4	32 ± 8	95 ± 29	182 ± 42	3
I ₃₀₇ N	-10 ± 5	-28 ± 12	-26 ± 6	41 ± 9	130 ± 30	337 ± 103	4
I ₃₀₇ W	-5 ± 2	-12 ± 1	-9 ± 5	66 ± 8	299 ± 21	889 ± 73	3
I ₃₀₇ R	-11 ± 5	-16 ± 3	14 ± 15	152 ± 38	364 ± 72	508 ± 33	3
I ₃₀₇ H	-2 ± 4	2 ± 5	5 ± 3	62 ± 5	139 ± 14	329 ± 19	3
I ₃₀₇ Q	3 ± 1	13 ± 5	23 ± 8	125 ± 22	249 ± 31	531 ± 66	4
R ₂₈₇ A	-5 ± 1	-41 ± 2	-54 ± 3	-63 ± 2	-53 ± 5	-8 ± 15	3
P ₂₉₄ A	-8 ± 1	-15 ± 1	-33 ± 3	-40 ± 3	-37 ± 3	-30 ± 5	5

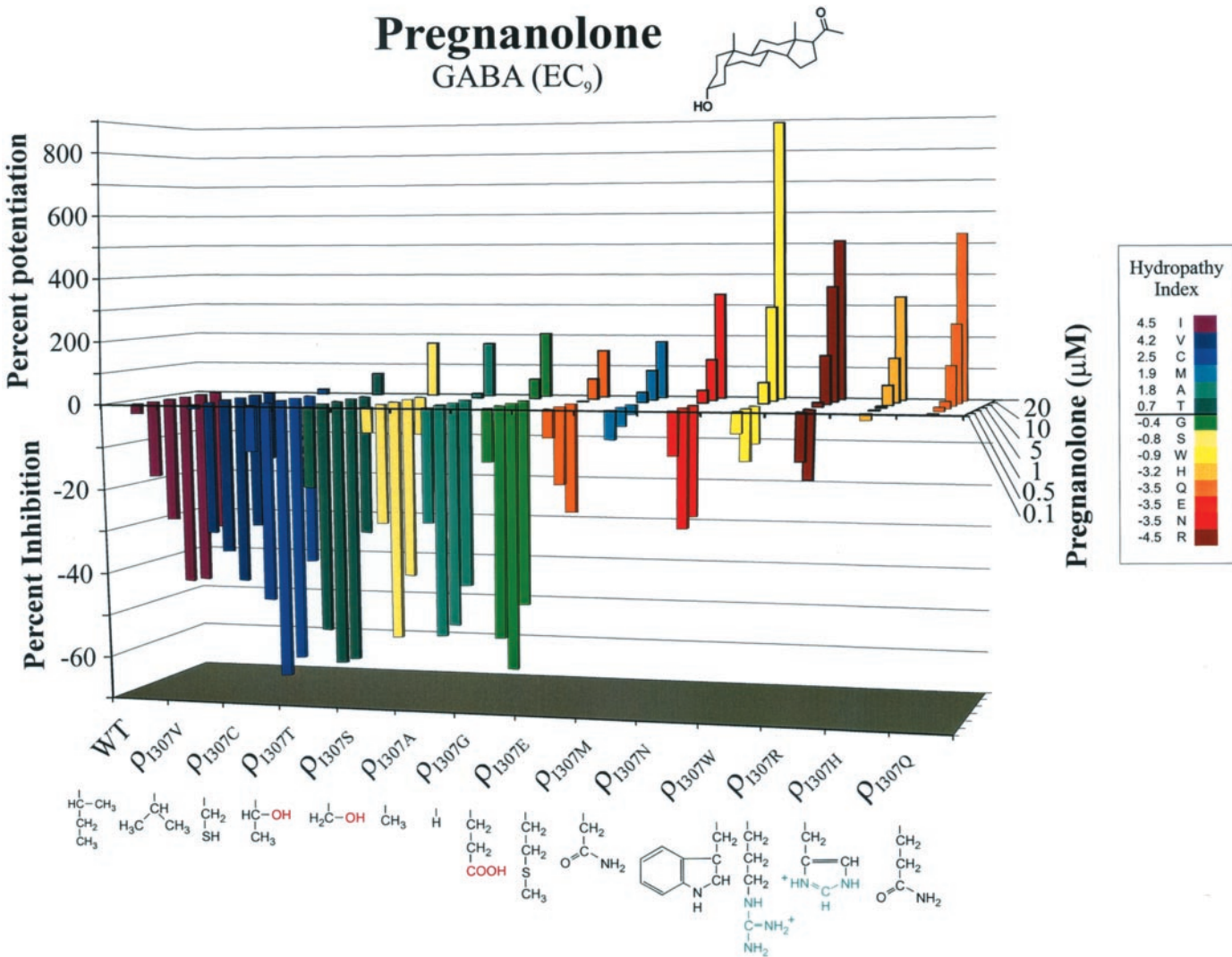


Fig. 4. Pregnanolone inhibits, potentiates, or elicits biphasic activity for Ile307 mutants. The bar graph represents the degree of pregnanolone modulation at 0.1, 0.5, 1, 5, 10, and 20 μM concentrations for 13 mutants and the wild-type in the presence of their respective EC₉ GABA (see Table 3 for S.E.M.). The hydropathy index of each amino acid is distinguished by the same color code used in the 5β-DHP experiments. The color spectrum progresses from violet, for the most hydrophobic, to red, representing the most hydrophilic amino acid. The data are arranged from left to right based on the concentrations at which pregnanolone modulation switched from inhibition to potentiation and then the degree of potentiation at 20 μM. The color change with respect to pregnanolone activity shows a relationship between the hydrophilicity of the side chain and the reversal of the direction of pregnanolone modulation.

investigated the effect of Ile307 mutation to Ser on the 5α -THDOC modulation. Figure 5 shows current traces and concentration-response relationships for 5α -THDOC-dependent modulation at 1, 5, 10, and 20 μM (EC_9 GABA) for the wild-type (gray lines) and ρ_{I307S} (blue lines). The 5α -THDOC produced a significantly greater potentiation for ρ_{I307S} than wild-type ρ_1 Rs. At 20 μM concentrations, 5α -THDOC potentiated the ρ_{I307S} GABA current ($820 \pm 79\%$)

greater than four times the wild-type ($198 \pm 9\%$). Next, we determined 5α -THDOC concentration-response relationships for ρ_{I307A} , ρ_{I307Q} , ρ_{I307C} , ρ_{I307W} , and ρ_{I307G} mutants to investigate whether any Ile307 mutation can change the positive direction of 5α -THDOC action. The substituted residues for these mutants represent a large variation in terms of amino acid side chain volume and hydrophobicity. Figure 6 shows the bar graph data for the 5α -THDOC-dependent modulation

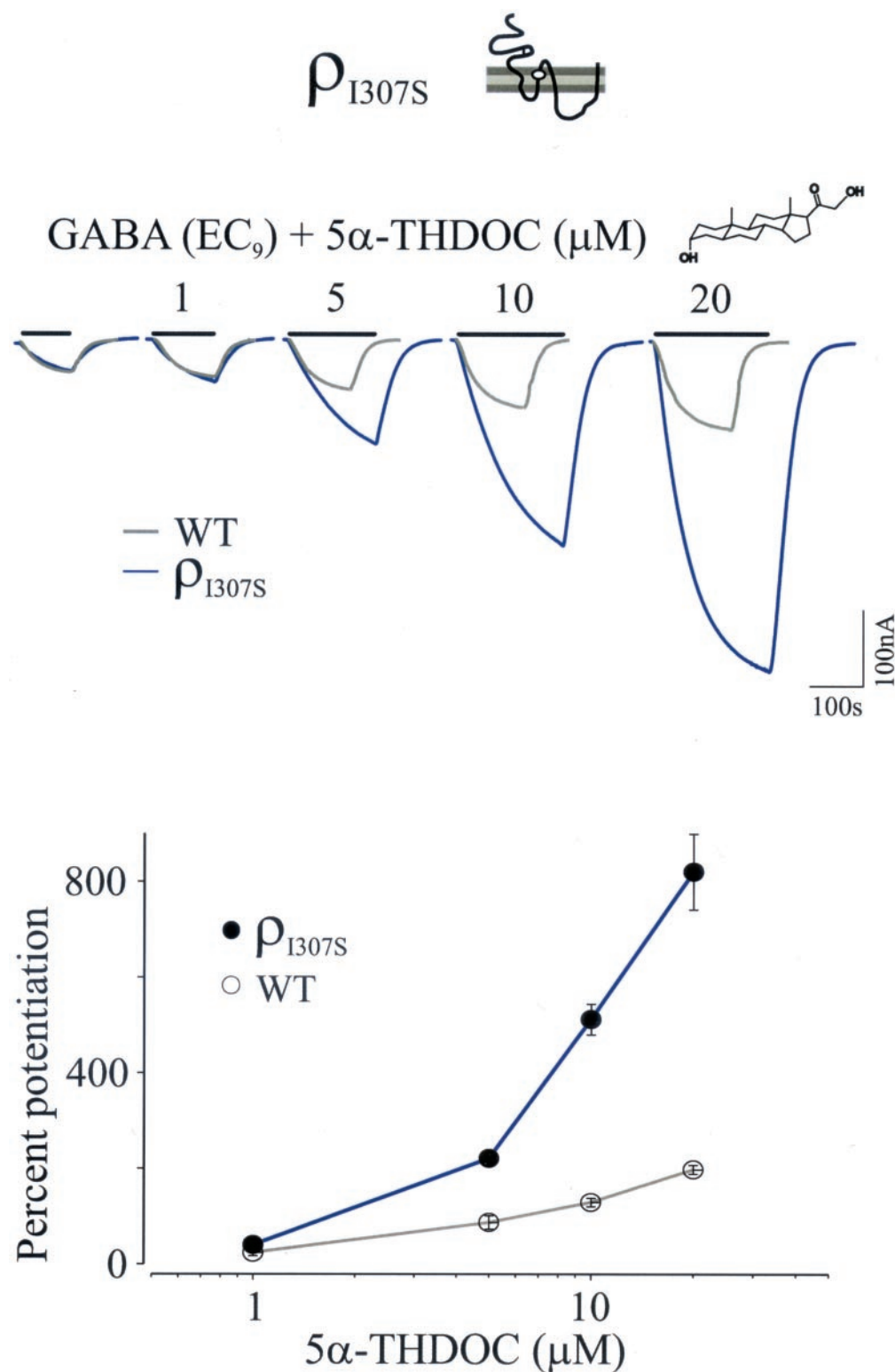


Fig. 5. The 5α -THDOC is a positive modulator of both wild-type and ρ_{I307S} R. The representative current traces (top) and concentration response relationship (bottom) for the 5α -THDOC-dependent modulation of EC_9 GABA at 1, 5, 10, and 20 μM for the wild-type (gray lines) and ρ_{I307S} R (blue lines) are shown. The control GABA current traces (for both wild-type and mutant) are shown at the same amplitude to allow a direct comparison between pregnanolone-dependent modulation of the wild-type and ρ_{I307S} R. Scale bar representing the current magnitude only applies to mutant current trace. Thick lines above each current trace represent the duration of GABA or GABA plus NS application for ρ_{I307S} traces only. The level of potentiation by 5α -THDOC significantly increased for ρ_{I307S} R compared with wild-type ρ_1 R.

of these mutants using the same color spectrum as in 5 β -DHP and pregnanolone experiments, with violet being the most hydrophobic and red the most hydrophilic (see also Table 4). The 5 α -THDOC only potentiated the GABA currents for ρ_{1307G} , ρ_{1307C} , ρ_{1307A} , ρ_{1307W} , and ρ_{1307Q} R across the concentration range. Moreover, the levels of potentiation for all mutants were significantly greater than the wild-type receptor-channels. For example, 5 α -THDOC (20 μ M) increased the respective EC₉ GABA current of ρ_{1307W} , ρ_{1307Q} , and ρ_{1307C} R by 740 ± 80 , 1293 ± 43 , and $1058 \pm 77\%$, respectively (wild-type = $198 \pm 9\%$). It is interesting that all substituted residues (Gly, Ser, Ala, Cys, Gln, Trp) producing greater potentiation than wild type were more hydrophilic than Ile (native residue).

Some of the values obtained for the 20 μ M potentiation were greater than the predicted GABA maximal (ρ_{1307Q} and ρ_{1307G}). If the conductance of the channel was unaltered by the mutation, the maximal potentiation based on EC₉ value should not exceed approximately 1100%. Previous studies (Morris et al., 1999) have demonstrated that variation in EC₉ calculation can significantly affect potentiation level (but not inhibition; see *Materials and Methods*). Although the variation in EC₉ calculation can be responsible for the above observation, the possibility that for some of these mutants 5 α -THDOC can potentiate GABA currents beyond the GABA-induced maximal cannot be ruled out.

The EC₅₀ value for 5 α -THDOC modulation could not be predicted, because even with the highest feasible concentration (20 μ M; see *Materials and Methods*), the maximal cur-

rent could neither be reached nor extrapolated. Nevertheless, the potentiation effect for all mutants and the wild-type became apparent between 1 and 5 μ M of 5 α -THDOC. Thus, mutation of the Ile307 to a number of different amino acids did not alter the concentration range at which the effects of 5 α -THDOC were discernible but caused a marked increase in the level of potentiation by 5 α -THDOC compared with the wild-type. However, in contrast to the mutational effect on 5 β -NS, none of the tested mutants changed the direction of modulation by 5 α -THDOC.

Preincubation with 5 α or 5 β Alone Produces an Equivalent Effect on NS Potentiation Kinetics. It is postulated that NSs, dependent on being a potentiator or an inhibitor, act on the ligand-gated ion channel via distinct sites (Prince and Simmonds, 1993; Park-Chung et al., 1999; Covey et al., 2000, 2001). Alternative binding sites would probably entail differences in apparent affinity of 5 α - versus 5 β -NS toward their respective targets. To test the above hypothesis (distinct sites), we investigated the effect of NS incubation alone on the activation time course of GABA plus NS for the mutated ρ_1 R. The 5 α - or 5 β -NSs at the highest feasible concentration (20 μ M) do not directly gate (are not agonist for) wild-type or the Ile307 mutant receptor-channels (data not shown). In these experiments, $t_{1/2}$ of activation was measured in the presence of GABA (EC₉) and NS (3 min of incubation), with or without a 6-min preincubation of the NS alone. The effects of 5 α preincubation on 5 α , those of 5 β on 5 β , and those of 5 α on 5 β modulation were determined using the above protocol. We chose ρ_{1307S} R and ρ_{1307W} R because

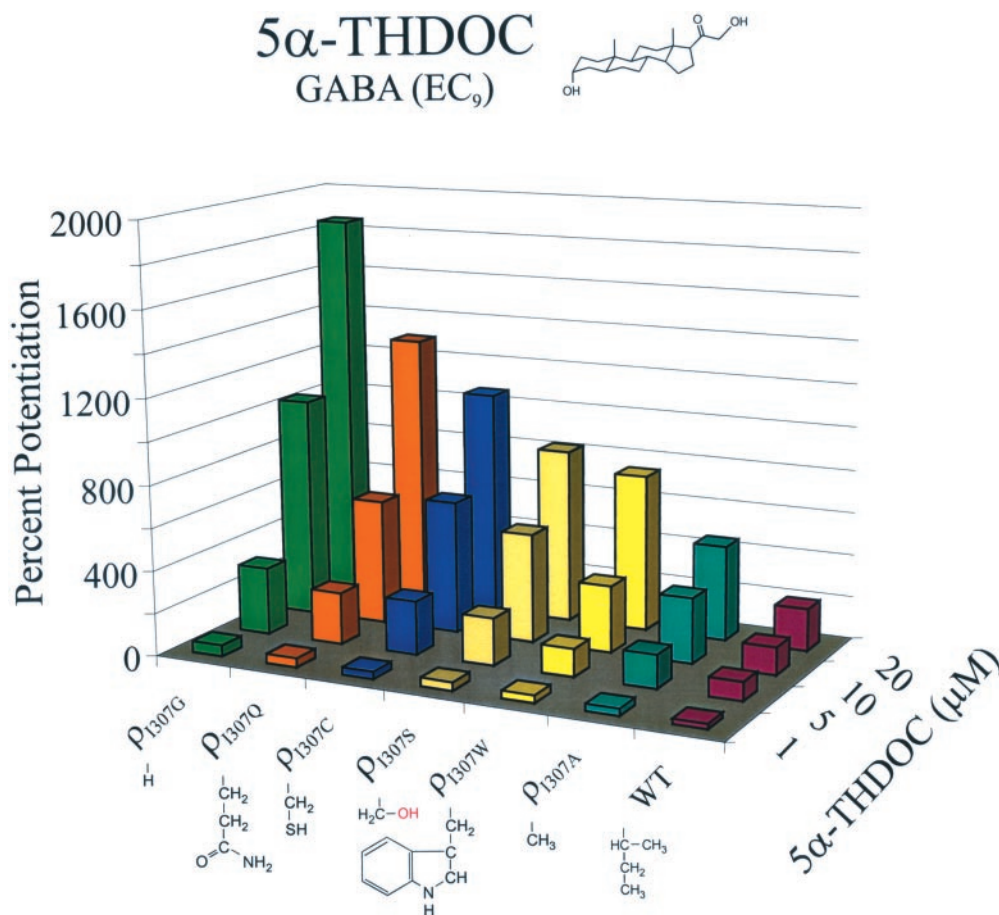


Fig. 6. The 5 α -THDOC is always a potentiator for Ile307 mutants. The 5 α -THDOC increased the GABA currents for ρ_{1307G} , ρ_{1307C} , ρ_{1307A} , ρ_{1307W} , and ρ_{1307Q} R across the concentration range. The levels of potentiation for all mutants were significantly greater than the wild-type receptor-channels. The hydrophathy index of each amino acid is distinguished by the same color code used in the previous figures, from violet (most hydrophobic) to red (most hydrophilic). For all tested Ile307 mutants, 5 α -THDOC only potentiated the corresponding GABA currents (EC₉).

they exhibit differences and similarities with respect to 5 β - and 5 α -NS action (see above). For example, 5 β -DHP is a potentiator of $\rho_{I307S}R$ but an inhibitor of $\rho_{I307W}R$ GABA current. Pregnanolone modulation is biphasic for $\rho_{I307S}R$ and $\rho_{I307W}R$ but shows differences in the details of its modulation. At all tested concentrations below 20 μM (0.1, 0.5, 1, 5, 10), pregnanolone inhibited the $\rho_{I307S}R$ but at only 20 μM potentiated the GABA current for the $\rho_{I307S}R$. In comparison, pregnanolone inhibited the $\rho_{I307W}R$ GABA current only at

TABLE 4

The 5 α -THDOC-dependent modulation of Ile307 mutants in the presence their respective EC₉ GABA

All data are presented as mean \pm S.E.M.

Subunit	1 μM	5 μM	10 μM	20 μM	n
ρI^*	24 \pm 7	87 \pm 14	129 \pm 9	198 \pm 9	6
I_{307G}	52 \pm 27	316 \pm 40	1036 \pm 116	1852 \pm 109	3
I_{307A}	32 \pm 3	155 \pm 11	305 \pm 24	446 \pm 37	3
I_{307W}	35 \pm 5	130 \pm 9	314 \pm 30	740 \pm 80	3
I_{307C}	34 \pm 9	252 \pm 19	624 \pm 48	1058 \pm 77	4
I_{307S}	40 \pm 2	221 \pm 1	512 \pm 32	820 \pm 79	6
I_{307Q}	43 \pm 7	244 \pm 25	589 \pm 10	1293 \pm 43	4

0.1, 0.5, and 1 μM , and at higher concentrations was a potentiator of $\rho_{I307W}R$ GABA current. On the other hand, 5 α -THDOC potentiated the GABA current for both $\rho_{I307S}R$ and $\rho_{I307W}R$ across the concentration range.

In the first series of experiments, $t_{1/2}$ of current activation for $\rho_{I307S}R$ was measured for coapplication of GABA (EC₉) and 10 μM 5 α -THDOC (3 min of incubation), with and without 6-min preincubation with 5 α -THDOC. Figure 7 shows the current traces for GABA application alone (2 times), GABA plus 10 μM 5 α -THDOC, and GABA plus 5 α -THDOC (10 μM) immediately after preincubation with 5 α -THDOC (10 μM). It is important to note that the holding current did not change during the 6-min preincubation with 5 α -THDOC alone (data not shown). Nevertheless, the preincubation with 5 α -THDOC decreased the activation $t_{1/2}$ of GABA plus 5 α -THDOC from 70.7 \pm 0.9 to 44.7 \pm 2.1 s ($n = 4$), thereby reducing the time interval by 26.0 s (see Table 5).

Using the above protocol, the effect of pregnanolone pretreatment (20 μM) on the activation $t_{1/2}$ of GABA and pregnanolone coapplication for both $\rho_{I307S}R$ and $\rho_{I307W}R$ was

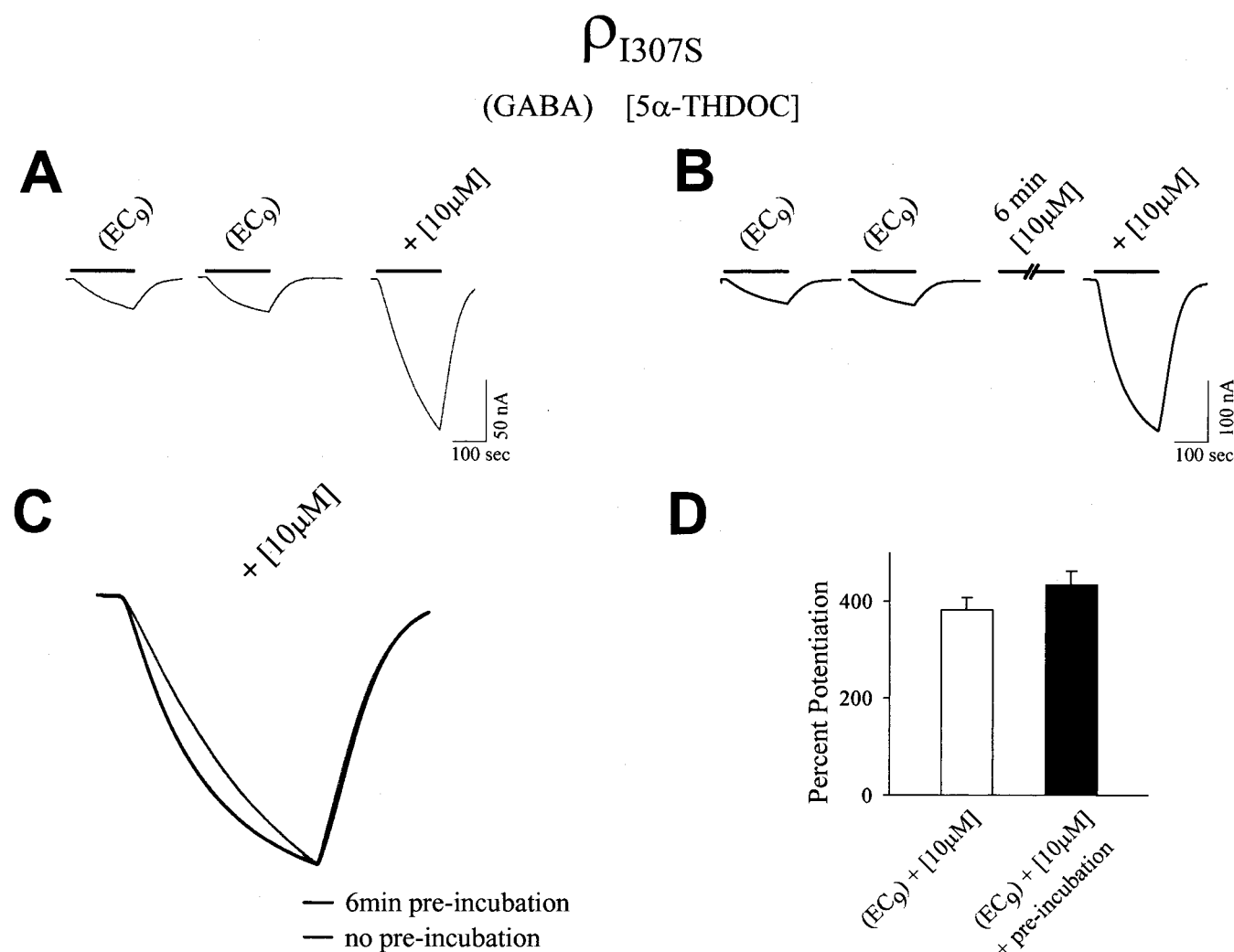


Fig. 7. Preincubation with 5 α -THDOC shortens the $t_{1/2}$ of activation for GABA plus 5 α -THDOC. A, two GABA-induced currents (3 min) from an oocyte expressing $\rho_{I307S}R$ followed by application of GABA plus 10 μM 5 α -THDOC (3 min). Note the slow rise of current induced by GABA plus 5 α -THDOC. B, current traces from two applications of GABA alone (3 min) expressing $\rho_{I307S}R$ (a different oocyte than that in A). The oocyte was then incubated with 10 μM 5 α -THDOC for 6 min followed immediately with application of GABA plus 10 μM 5 α -THDOC (3 min). C, the difference in the rate of activation between GABA plus 10 μM 5 α -THDOC and GABA plus 10 μM 5 α -THDOC after the 5 α -THDOC preincubation. D, the 5 α -THDOC preincubation effect on the percentage potentiation of the subsequent 5 α -THDOC and GABA application.

examined. The $t_{1/2}$ of pregnanolone plus GABA was nearly identical for both mutants. The corresponding half-lives for $\rho_{1307S}R$ and $\rho_{1307W}R$ were 68.0 ± 1.15 and 71.5 ± 2.3 s ($n = 4$), respectively. The preincubation with pregnanolone (20 μ M) for 6 min decreased the $t_{1/2}$ of activation by very similar intervals for both mutants. After the treatment with pregnanolone alone, the pregnanolone potentiation half-life was reduced to 44.0 ± 1.15 and 44.6 ± 2.7 s for $\rho_{1307S}R$ and $\rho_{1307W}R$, respectively.

If 5α - and 5β -NSs modulated the receptor-channels through alternate binding sites, pretreatment with one NS (e.g., 5α -THDOC) would be expected to produce a differential effect on the activation kinetics of the other (e.g., pregnanolone). The impact of 5α -THDOC (10 μ M) pretreatment on the $t_{1/2}$ of pregnanolone (20 μ M) potentiation was determined using the above protocol for both $\rho_{1307S}R$ and $\rho_{1307W}R$ (Table 5). Preincubation for 6 min with 5α -THDOC decreased the activation $t_{1/2}$ for pregnanolone by equivalent intervals for both mutants. Moreover, these shifts in activation time were highly similar to those determined for the preincubational effect of 5α on 5α $t_{1/2}$ or the effect of 5β on 5β $t_{1/2}$. Pregnanolone or 5α -THDOC preincubation reduced the activation $t_{1/2}$ of pregnanolone modulation of the GABA current for either $\rho_{1307S}R$ or $\rho_{1307W}R$ by approximately 25 s (Table 5).

For the $\rho_{1307S}R$, preincubation with 5α alone increased the percentage potentiation for 5α -THDOC from 348 ± 37 to $433 \pm 27\%$. In comparison, after 5β preincubation, potentiation nearly doubled for pregnanolone-dependent modulation (from $182 \pm 46\%$ to $360 \pm 29\%$). Moreover, the significant increase in potentiation level for pregnanolone modulation was independent of whether the preincubation was with a 5α - or a 5β -NS. Thus, preincubation with 5α -THDOC closely mimicked that of pregnanolone upon pregnanolone-dependent modulation, not only in terms of impact on the activation kinetics but also with respect to the effect on potentiation.

Discussion

Within a ρ_1R model system, we have demonstrated that a single amino acid mutation at Ile307 can induce a reversal in modulation (inhibition to potentiation) by 5β - but not by 5α -NSs. For Ile307 mutants, 5β -DHP produced a range of modulation from inhibition to potentiation. In comparison, depending on the mutation, pregnanolone remained an inhibitor, transformed into a potentiator, or converted to inhibitor and potentiator of GABA currents based on concentration. The amino acid side chain property/activity plot demonstrated a correlation between 5β -NS direction of activity and side-chain hydrophobicity. On the other hand, the ρ_1 307 mutants did not change the mode of modulation by 5α -THDOC but caused a significant increase in the potentiation level. Despite the severe impact on the degree or/and mode of modulation, none of the 307 mutations significantly affected the NS concentration producing the initial response. Moreover, preincubation of Ile307 mutants with either 5α or 5β alone produced an equivalent effect on the activation time course of NS-dependent modulation.

Inconsistency with a Binding Site or Cavity Hypothesis for the NS Action at the Position 307. The prevailing view for molecular actions of NSs and anesthetics is that they interact with defined binding sites or cavities to exert their effects. These current models propose that anesthetics may

fill a pocket with specific dimensions and thereby alter the receptor-channel gating and/or agonist binding activity (Wick et al., 1998; Ye et al., 1998; Koltchine et al., 1999; Ueno et al., 2000; Covey et al., 2001; Jenkins et al., 2001).

Our data on NS modulation of ρ_1R with respect to position 307 is inconsistent with the binding site(s) or fixed volume cavity hypotheses for the following reasons:

1. The simple mass action principle predicts that subtle impairment of a ligand binding site can be overcome with higher concentrations of ligand; conversely, enhancement of the binding site would require a lower concentration of the ligand to produce an equivalent effect. For example, conservative mutation of the GABA binding domain within the receptor site causes a marked impact on potency. That is to say, mutation of the Tyr157 to Phe (conserving the aromatic ring) or to Ser (conserving the hydroxyl group) within the β_2 subunit only of the GABA_A receptor ($\alpha_1\beta_2\gamma_2$) impairs the GABA-dependent action by approximately 2 and 3 orders of magnitude, respectively (EC_{50} of 45.8 ± 3.6 μ M for wild type compared with 2570 ± 646 for $\alpha_1\beta_{Y157F}\gamma_2$ and $43,580 \pm 6800$ μ M for $\alpha_1\beta_{Y157S}\gamma_2$; Amin and Weiss, 1993). Compared with the mutation effect on the GABA binding site within receptor domain of $\alpha_1\beta_2\gamma_2$, mutations of Ile307 within the ρ_1R (and with a 5-fold degeneracy because of the homo-oligomeric nature of ρ_1R) to amino acids with opposite charges (Glu versus Arg) or dramatically different sizes (Gly versus Trp) respond to NSs within the same and relatively small concentration range. For instance, the effect of 5β -DHP with respect to inhibition or potentiation is discernible between 1 to 20 μ M for all mutants, and the concentration initiating the effect is around 5 μ M. If there were a moderate impairment or enhancement within the 5β -DHP binding domain caused by Ile 307 mutation, 5β -DHP would either produce no effect on the GABA currents (shift of sensitivity to higher concentrations) or create nearly maximal modulation (shift of the concentration response to lower concentrations) within such a small concentration range (1 - 20 μ M). Earlier studies (Amin 1999) have shown that the mutation of Trp328 to a number of hydrophobic

TABLE 5

Pre-incubation with 5α or 5β NS shortens the $t_{1/2}$ of activation for the NS and GABA by equivalent intervals

All data are presented as mean \pm S.E.M.

Mutant	Pre-incubation with	$t_{1/2}$ of GABA +	$\Delta t_{1/2}$
ρ_{1307S}	5α -THDOC	5α -THDOC	
	0 min	70.7 \pm 0.7 s	26.0s
	6 min	44.7 \pm 2.1 s	
ρ_{1307S}	Pregnanolone	Pregnanolone	
	0 min	68.0 \pm 1.15 s	24.0s
	6 min	44.0 \pm 2.0 s	
ρ_{1307S}	5α -THDOC	Pregnanolone	
	0 min	68.0 \pm 1.15 s	24.0s
	6 min	44.0 \pm 1.15 s	
ρ_{1307W}	Pregnanolone	Pregnanolone	
	0 min	71.0 \pm 2.3 s	26.4s
	6 min	44.6 \pm 2.7 s	
ρ_{1307W}	5α -THDOC	Pregnanolone	
	0 min	71.0 \pm 2.3 s	28.0s
	6 min	43.0 \pm 5.0 s	

residues can impart pentobarbital sensitivity to ρ_1 R. As with Ile307 mutants, Trp328 mutants also exhibit similar sensitivity to pentobarbital direct activation. As a group, these experiments demonstrate a dramatic contrast between the mutational effect on a ligand-binding site like GABA and a key site for NS (or pentobarbital) action.

2. In the last decade, multiple studies have pointed to the importance of residues 307 and 328 within ρ_1 R (as well as other residues and their equivalents in GABA_A receptor subunits) for a number of anesthetics (Walters et al., 2000; Belelli et al., 1997, 1999; Mihic et al., 1997; Amin, 1999). All these residues that are important for anesthetic action are found within the TM domains, yet none is found within the receptor domains. Similar to this study, mutations of these important "anesthetic" residues within the TM domains showed a paradigm completely distinct from the mutational analysis of a typical agonist-dependent activation domain like GABA (see above).
3. A binding pocket(s) or a cavity within a protein structure

has defined dimensions and would be constrained by the side chains of its amino acid constituents. Mutations of Ile307 to a more hydrophilic residue at position 307 can markedly affect the level of inhibition or produce potentiation. Pregnanolone can inhibit or potentiate the same mutated receptor-channel depending on the concentration. The biphasic activity by pregnanolone can occur with Ile307 mutations to residues representing opposites within the volume spectrum (Gly or Trp), and opposites within the charge spectrum (Arg and Glu). It seems highly unlikely that amino acids with opposite charge, or of significantly different size, produce equivalent effects within the architecture of a binding site or cavity.

4. Earlier reports (Williams and Akabas, 2002) with the intravenous anesthetic propofol and GABA_ARs using Cys scanning of the TM3 demonstrated that the profiles of the TM3 movement during GABA- versus GABA and propofol-dependent activation were different. Because the

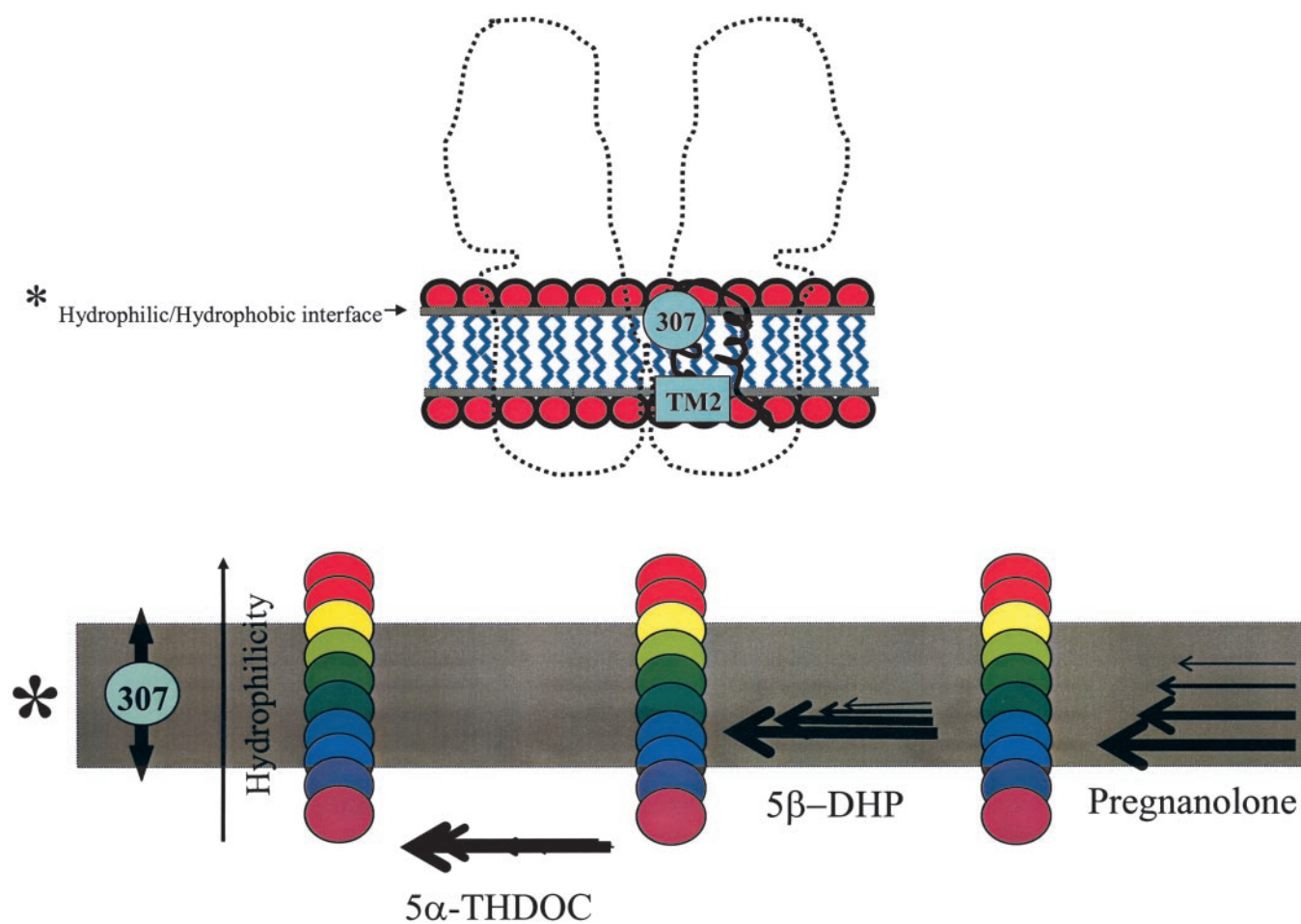


Fig. 8. A model representation of 5α and 5β -NS action upon a ligand-gated ion channel. In this model, the position 307 is a sensor site and each NS exerts unique force dependent on minute structural differences and concentration. Amino acid 307 is positioned at the interface of hydrophobic/hydrophilic boundary of the membrane bilayer. Within the wild-type ρ_1 R, Ile307 may favor the hydrophobic domain (because Ile has the highest hydrophobicity index). The amino acid color code ranges from violet (most hydrophobic) to red (most hydrophilic). All residues within the 307 mutational series are more hydrophilic than the Ile. Thus, mutations of residue 307 to any residue may support the movement of this position toward the hydrophilic side to reduce free energy. Transmembrane domains of the receptor-channel crossing the membrane can induce gross perturbation (or disorder) within the lipid bilayer. NSs, in this scenario, can incorporate within these perturbed membrane domain closely surrounding the receptor-channel at or near the protein/membrane interface and dependent on minor structural differences and concentration induce a unique three-dimensional profile on the sensor site. The position and length of the arrow represent the distinct depth and the pressure that a given NS may exert upon the gating component. If the sensor site is above the arrow, the forces acting upon the channel gate will translate into potentiation. On the other hand, if the sensor site is below the arrow, the action of the NS on the ion channel will be inhibitory.

GABA_ARs transit between a number of open and closed states (Weiss and Magleby, 1989; MacDonald and Twyman, 1992; Maconochie et al., 1994), creation of variable size cavities to accommodate propofol was predicted as channels move from one state to another. Here, preincubation with 5 α - or 5 β -NS produced equivalent effects on the activation time course of either 5 α - or 5 β -NS-dependent modulation. The effect of 5 α -THDOC preincubation on the pregnanolone activation time course ($t_{1/2}$) was nearly identical to the effect of 5 α -THDOC preincubation on 5 α -THDOC $t_{1/2}$ and that of pregnanolone on pregnanolone $t_{1/2}$. These effects occurred in the absence of channel activity because the 5 α - or 5 β -NS does not directly gate the ρ_1 R. The presence of alternative binding sites for NSs (inhibition versus potentiation) with a fixed volume predicts differential affinity and therefore a disparate impact on the $t_{1/2}$ of activation. In addition, variably sized binding sites require channel opening to accommodate the ligand. Our findings of nearly identical effects of preincubation with 5 α - or 5 β -NS on the subsequent channel activity seem inconsistent with alternative or variable binding site hypothesis.

Is Ile 307 Involved in Coupling the NS Binding Event to the Channel Gating? In addition to the impact on NS-dependent modulation, sensitivity of structurally diverse anesthetics such as pentobarbital and benzodiazepines can be imparted upon the ρ_1 R using the mutation of Ile 307 and/or Trp328. It has been postulated that position 307 may play an essential role in the transduction pathway, between the NS binding and the channel gating. At such a crucial linkage site, one may expect imposing constraints at the level of the amino acid side chain. In addition, it is difficult to reconcile that a single mutation between the binding and the gating can result in reversing the propagation event from inhibiting to facilitating channel gating (from inhibition to potentiation) or act as an 'linkage' for both inhibition and potentiation (pregnanolone).

A Proposed Model for NS Action. How does a single mutation reverse polarity for modulation of 5 β -DHP but not for that of 5 α ? What is the mechanism for pregnanolone biphasic modulation? We propose a model in which 1) the mutation of Ile307 can influence the positioning of a sensor site and 2) each NS exerts a unique three-dimensional force dependent on minute structural differences as well as concentration (Fig. 8). The TM2 domain contains important gating features for the ion channel, and Ile307 is an agile component of this module (Unwin, 1995; Horenstein et al., 2001). Amino acid 307 is located five amino acids from the extracellular surface, at the interface of the hydrophobic/hydrophilic boundary of the membrane bilayer (Fig. 8, asterisk). Within the wild-type ρ_1 R, Ile307 favors the hydrophobic domain (Ile has the highest hydrophobicity index). However, mutations of residue 307 to any residue more hydrophilic than Ile may support the movement of this sensor site toward the energetically more favorable hydrophilic environment. Transmembrane domains of the receptor-channel (crossing the membrane) can induce gross perturbation (disorder) within the lipid bilayer. NSs, in this scenario, can incorporate within these perturbed membrane domains closely surrounding the receptor-channel at or near the protein/membrane interface and dependent on minor structural differences and concen-

tration induce a unique three-dimensional energy profile on the sensor site. The position and length of the arrow within the model represents the distinct depth and the pressure that a given NS may exert upon the gating component. If the sensor site is above the arrow, the forces acting upon the channel gate will translate into potentiation. On the other hand, if the sensor site is below the arrow, the action of the NS on the ion channel will be inhibitory (see Cantor, 1997 for another rendition). This model can account for the mutational effect reversing the action of 5 β -DHP. Furthermore, it explains how pregnanolone can inhibit or potentiate a given mutant based on its concentration. Finally, such a model foresees the presence of a neutral zone for the sensor site where the NS window of impact may have little effect on the GABA-dependent modulation. In accordance with this view, 5 β -DHP showed little effect upon certain Ile307 mutants such as ρ_{1307T} (see *Results*).

In a homo-oligomeric ρ_1 R model system, we demonstrated that mutation of a key residue within TM2 can induce a profound effect on the NS mode and degree of modulation yet produce little change in the apparent sensitivity of the receptor-channel to the NS. Preincubation with either a 5 α - or 5 β -NS produced an equivalent effect on the potentiation time course. Future experimentation, by means of single channel analysis as well as high-resolution structural mapping of the NS interaction site with its target, will be needed to further test this model.

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Address correspondence to: Jahanshah Amin, PhD, University of South Florida, College of Medicine, Departments of Pharmacology and Therapeutics, MDC Box 9, 12901 Bruce B. Downs Blvd., Tampa, FL 33612. E-mail: jamin@hsc.usf.edu