

The Neurosteroids Dehydroepiandrosterone Sulfate and Pregnenolone Sulfate Inhibit the UNC-49 GABA Receptor through a Common Set of Residues

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ABSTRACT

Neurosteroids are endogenous neuromodulators that bind and allosterically regulate GABA_A receptors. Residues were recently identified in the first transmembrane domain (M1) of GABA_A receptor subunits that are important for neurosteroid modulation. We are studying the inhibition of GABA_A receptors by sulfated neurosteroids. One of these neurosteroid, pregnenolone sulfate (PS), depends on six identified M1 residues to inhibit the UNC-49 GABA receptor, a homomeric GABA receptor from *Caenorhabditis elegans* that is homologous to the mammalian GABA_A receptor. Here, we investigate the inhibition of the UNC-49 GABA receptor by another sulfated neurosteroid, dehydroepiandrosterone sulfate (DHEAS). DHEAS is identical to PS except that it contains a carbonyl oxygen instead of an acetyl group at C17 on the steroid D ring. UNC-49 mutations

that affect PS inhibition had broadly parallel effects on DHEAS, suggesting the two neurosteroids act through similar mechanisms. However, certain M1 mutations affected DHEAS differently than PS. Considering that first, the D ring contains the only structural difference between PS and DHEAS, and second, the strongest chemical and steric effects of a mutation are likely to be felt in the local environment of the altered residues, this result implies that the steroid D ring may contact M1 near the mutated residues. This possibility is interesting because current models of neurosteroid interactions with GABA_A receptors, based on pregnane steroids, suggest that the steroid A ring binds M1, whereas the D ring binds M4. Our findings suggest that there may be considerable diversity in the way different classes of neurosteroids interact with GABA_A receptors.

The GABA_A receptor is the principal inhibitory neurotransmitter receptor in the brain. Important anxiolytic, anticonvulsant, and sedative/hypnotic drugs bind to specific sites on the GABA_A receptor and allosterically potentiate its function (Macdonald and Olsen, 1994). The GABA_A receptor is also a target of the neurosteroids, which are endogenous brain steroids with neuromodulatory activities. Some neurosteroids enhance GABA_A receptor function and act as anxiolytics and anticonvulsants (e.g., allopregnanolone and tetrahydrodesoxycorticosterone). Others inhibit GABA_A receptor function and can improve cognitive function and reduce seizure threshold (e.g., the sulfated neurosteroids PS and DHEAS) (Belelli and Lambert, 2005). Therefore, the neurosteroid site is a potential target for new drugs to modulate GABA_A receptor function.

Structure-activity studies and structure-function studies

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have been valuable for understanding the interaction between neurosteroids and GABA_A receptors. The structure-activity approach has highlighted the significance of the functional group attached to the 3-carbon and the stereochemistry of the 5-carbon in the steroid A ring, and the functional group attached to the 17-carbon in the D ring (Morris et al., 1999; Covey et al., 2001; Hamilton, 2002; Morris and Amin, 2004; Akk et al., 2007). Structure-function studies have begun to identify important GABA_A receptor residues for neurosteroid sensitivity, demonstrating particular importance for the first transmembrane domain (M1) (Akk et al., 2001; Chang et al., 2003; Morris and Amin, 2004; Hosie et al., 2006; Wardell et al., 2006). For example, mutating six M1 residues in conjunction with one residue in the linker between the second and third transmembrane domains (the M2-M3 linker) in various combinations increased the PS sensitivity of the *Caenorhabditis elegans* UNC-49 GABA_A receptor over a 57-fold range (Wardell et al., 2006). The availability of a panel of receptor mutants now allows us to test multiple steroids that have different substituents at the 17-carbon (C17) against multiple receptors that contain minor but

ABBREVIATIONS: PS, pregnenolone sulfate; DHEAS, dehydroepiandrosterone sulfate; M, transmembrane domain.

functionally significant sequence alterations. Such a matrix analysis can indicate which amino acid side chains are necessary for the receptor to discriminate between different neurosteroids. Reciprocally, it can demonstrate which steroid substituents at C17 need to be present for a particular receptor mutation to have its effect. By identifying these interdependencies, we can begin to map the amino acids that interact with particular steroid moieties, thus refining our understanding of the neurosteroid-receptor interaction.

In this study, we have used 16 mutant variants of the *C. elegans* UNC-49 GABA_A receptor to compare the mechanisms of two neurosteroids, DHEAS and PS. This is an interesting comparison because the two steroids are identical except that DHEAS has a carbonyl oxygen at C17 of the steroid D ring, whereas PS has an acetyl group (Fig. 1). Residues that, when mutated, have a parallel effect on PS and DHEAS are likely to mediate binding contacts or conformational changes that can be attributed to shared structural features (i.e., the steroid A, B, and C rings). Alternatively, residues that have differential effects when mutated are more likely to play a role specific to the structural features that are different (i.e., the C17 functional group). We observe that most receptor mutations caused parallel shifts in potency for PS and DHEAS, but that certain M1 mutations had differential effects. We conclude that the inhibitory mechanisms of the two steroids are similar, and we identify a role for M1 residues in recognizing the C17 functional group of sulfated neurosteroids.

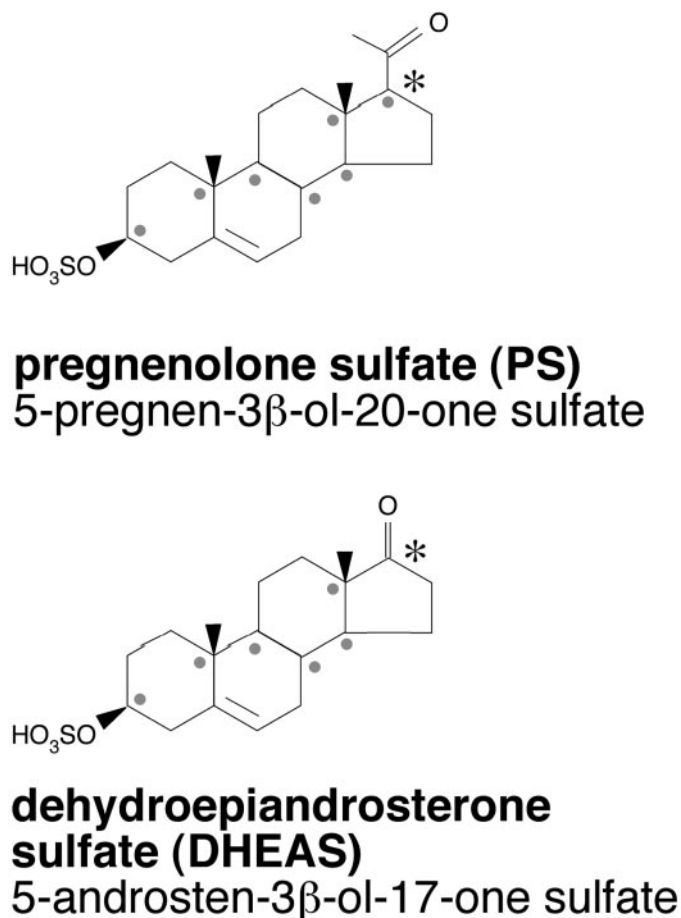


Fig. 1. Structures of PS and DHEAS. Asterisk indicates carbon-17; gray circles indicate chiral carbons.

Materials and Methods

Electrophysiology. Plasmids containing wild-type and mutagenized *C. elegans* GABA_A receptor subunits were linearized and transcribed in vitro using T3 polymerase (mMessage mMachine T3 kit; Ambion, Austin, TX). UNC-49B was injected into *Xenopus laevis* oocytes at 300 ng/μl either alone or in a mixture with 300 ng/μl UNC-49C. All other *C. elegans* subunits were injected alone at 300 ng/μl. The injection volume for all constructs was 50.6 nl. Oocytes were analyzed using two-electrode voltage-clamp electrophysiology with a GeneClamp 500 amplifier (Molecular Devices, Sunnyvale, CA), as described previously (Bamber et al., 2003). Cells were voltage-clamped at −60 mV. All recordings were performed at room temperature.

All steroid inhibition was measured at the GABA EC₅₀ concentration. Previously published GABA EC₅₀ values (Wardell et al., 2006) were used for all recordings except UNC-49B, UNC-49B/C, and QF-R. UNC-49B and UNC-49B/C GABA EC₅₀ values are highly variable (Bamber et al., 2003), and they were measured for each batch of oocytes. We also observed variability in the QF-R GABA EC₅₀, and a value of 20.6 μM (*n* = 3) was used in this study. No other GABA dose responses performed in this study differed significantly from their previously published values.

Inhibition for DHEAS, PS, and their corresponding enantiomers was measured for each steroid by preapplying the steroid for 20 s and then coapplying the steroid and GABA. We determined that 10- and 20-s DHEAS preapplications produced the same amount of inhibition, suggesting that the amount of DHEAS bound to the receptor was no longer changing at the time that GABA was applied (data not shown). Generally, DHEAS, PS, and their corresponding enantiomers evoked little or no direct response. In this study, the direct effect of PS on T257F/S264A was the only exception, and the observed effect was as described previously (Wardell et al., 2006). Where PS direct effects were observed, GABA-evoked peak currents were measured from the baseline in the presence of PS rather than the baseline before PS application. We routinely verified that currents recovered to their original magnitudes once the inhibitors had been removed. Currents reported in this study are the peak currents observed upon GABA application.

GABA and PS were obtained from Sigma-Aldrich (St. Louis, MO). DHEAS was obtained from Steraloids (Newport, RI). Enantiomers of DHEAS and PS were synthesized as described previously (Nilsson et al., 1998). GABA was prepared as a 1 M stock in water, and it was stored at −20°C for up to 1 year. DHEAS, PS, and their corresponding enantiomers were prepared as 10 mM stocks in dimethyl sulfoxide. The final dimethyl sulfoxide concentration was 1% at the maximal steroid concentrations tested, which does not inhibit or enhance GABA-evoked currents from UNC-49 GABA receptors (Wardell et al., 2006).

Data Analysis. For each subunit tested, the GABA EC₅₀ concentrations were determined by performing GABA dose-response curves and fitting them with the following equation: $I = I_{\max} / [1 + 10^{(\log EC_{50} - [\text{agonist}]) \times n}]$, where *I* is current at a given GABA concentration, *I*_{max} is current at saturation, EC₅₀ is the GABA concentration required to produce half-maximal current, and *n* is the slope coefficient. GABA dose-response curves were fit using Prism software (GraphPad Software Inc., San Diego, CA).

All steroid dose-response curves were fitted using the equation: $I_{\text{inh}} / I_{\text{inh-}} = 1 / [1 + (I_{\text{inh}} / IC_{50})^n + 1]$, where *I*_{inh} / *I*_{inh-} is the current in the presence of inhibitor (DHEAS, PS, or corresponding enantiomer) relative to GABA alone, IC₅₀ is the concentration of inhibitor required to block 50% of the current, and *n* is the slope coefficient. Steroid dose-response curves were fit using GraphPad Prism.

Error values presented in the text and error bars in the figures are standard error of the mean. *P* values were calculated using two-tailed Student's *t* tests unless otherwise stated.

Results

Strategy to Compare the Residues Required for PS and DHEAS Inhibition. In an earlier study, residues required for PS inhibition of the *C. elegans* UNC-49 GABA_A receptor were identified. The *unc-49* gene encodes two GABA_A receptor subunits by alternative splicing, designated UNC-49B and UNC-49C (Bamber et al., 1999). These subunits share identical extracellular amino terminal sequences, but they differ in the transmembrane domain region (Fig. 2A). UNC-49B forms a PS-insensitive homomeric receptor. UNC-49C is not functional alone, but it can assemble with UNC-49B to form a PS-sensitive heteromeric receptor, suggesting that UNC-49C residues mediate PS inhibition. The important UNC-49C residues were identified in three steps. First, UNC-49C sequences were swapped

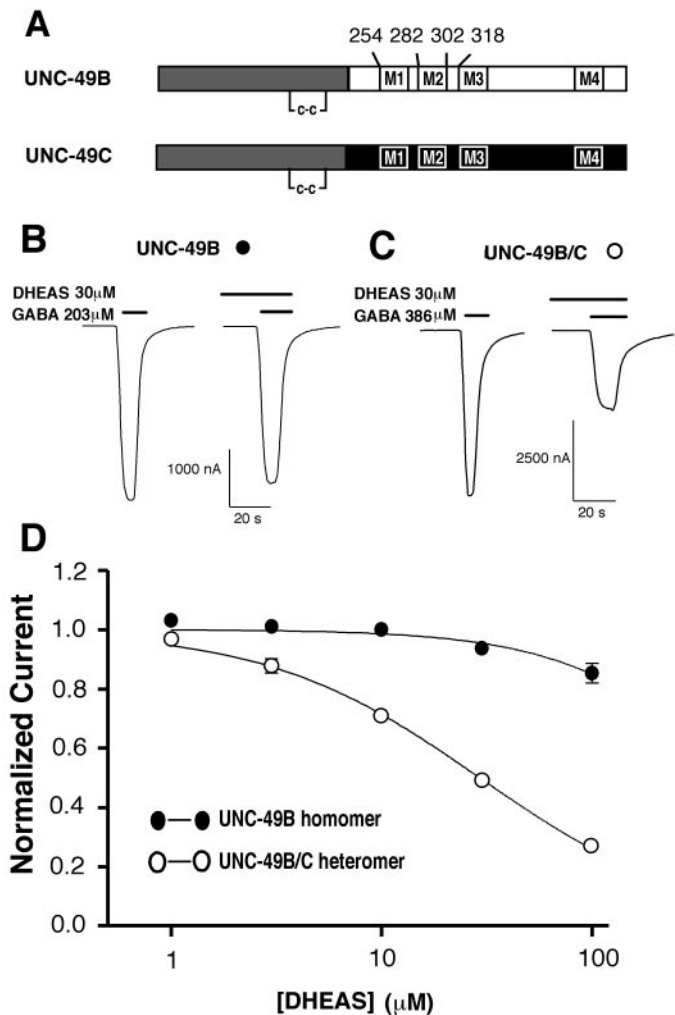


Fig. 2. The UNC-49B/C heteromer is more sensitive to DHEAS inhibition than the UNC-49B homomer. **A**, structure of the UNC-49B and UNC-49C subunits showing shared amino-terminal region (gray), and unique transmembrane domain regions (white and black for UNC-49B and UNC-49C, respectively). c-c indicates conserved disulfide loop, and M indicates transmembrane domain. **B** and **C**, DHEAS inhibition of GABA-evoked currents from *Xenopus* oocytes expressing UNC-49B homomeric GABA receptors (**B**) or UNC-49B/C heteromeric receptors (**C**). DHEAS was pre-applied for 20 s before coapplication of DHEAS and GABA (at EC₅₀). **D**, DHEAS dose-response curves for UNC-49B homomers and UNC-49B/C heteromers. Peak currents evoked by EC₅₀ GABA plus DHEAS normalized to currents evoked by EC₅₀ GABA alone are plotted against DHEAS concentration. Error bars represent S.E.M. ($n = 4$ oocytes for each receptor).

into UNC-49B, resulting in chimeric subunits that formed PS-sensitive homomers. Both the M1 and the M2-3 linker together were necessary for maximal PS sensitivity. Second, residues within these domains that were conserved in PS-sensitive subunits and divergent in UNC-49B were examined. These residues accounted for some of the PS sensitivity. Third, systematic mutagenesis identified the remaining necessary residues (Wardell et al., 2006). Here, we examine the DHEAS sensitivity of selected mutants generated in that previous study to compare its mechanism of inhibition to that of PS.

The UNC-49C Subunit Confers Sensitivity to DHEAS Inhibition. To determine whether the inclusion of the UNC-49C subunit increases DHEAS sensitivity, we compared DHEAS inhibition of UNC-49B homomers and UNC-49B/C heteromers. The UNC-49 receptors were expressed in *X. laevis* oocytes and analyzed by two-electrode voltage-clamp analysis. Currents evoked by EC₅₀ GABA plus DHEAS (1–100 μ M) were compared with currents evoked by EC₅₀ GABA alone (see *Materials and Methods*). DHEAS had little inhibitory activity at UNC-49B homomers (Fig. 2, B and D; Table 1), but it was a far stronger inhibitor of UNC-49B/C heteromers (Fig. 2, C and D; Table 1). Therefore, similar to PS, the UNC-49C subunit increases DHEAS sensitivity, suggesting that structural determinants of DHEAS sensitivity are contained within the UNC-49C subunit.

DHEAS Sensitivity Is Increased by UNC-49C Residues in M1 and Positive Charge in the M2-3 Linker. Significant increases in PS sensitivity of the UNC-49B receptor were achieved by mutating asparagine 305 in the M2-3 linker to arginine and swapping in the M1 domain of UNC-49C (Wardell et al., 2006). We tested whether these same changes had parallel effects on DHEAS sensitivity. First, we examined DHEAS inhibition in UNC-49B (N305R) homomers. Similar to PS, this mutation had little effect at lower DHEAS concentrations, but it increased sensitivity at higher concentrations (30 and 100 μ M; Fig. 3, A and C; Table 1). We then tested a receptor containing the N305R mutation plus the complete UNC-49C M1 domain

TABLE 1
DHEAS sensitivity of UNC-49 GABA_A receptors
 $n = 4$ unless otherwise indicated.

Subunit	DHEAS IC ₅₀ μ M	Slope Coefficient
UNC-49B	500 ⁱ	1.1 \pm 0.3
UNC-49B/C	29.2 \pm 1.4	0.9 \pm 0.1
N305R	98.8 \pm 3.3	1.5 \pm 0.1
M1-R ^a	27.2 \pm 5.2	1.1 \pm 0.1
QF-R ^{a,b}	26.0 \pm 2.0	1.1 \pm 0.02
X ^c	43.3 \pm 1.2	0.1 \pm 0.04
Y ^d	47.8 \pm 12.4	0.7 \pm 0.03
XY ^d	14.6 \pm 2.1	1.51 \pm 0.2
Y(T257F) ^d	20.2 \pm 2.0 ($n = 6$)	0.8 \pm 0.1 ($n = 6$)
Y(M258L) ^e	30.6 \pm 0.8	0.9 \pm 0.03
Y(S264A) ^f	107.8 \pm 8.8	0.9 \pm 0.1
Y(T257F/M258L) ^e	51.0 \pm 6.6	0.8 \pm 0.1
Y(T257F/S264A) ^e	107.6 \pm 23.1	0.1 \pm 0.06
Y(T257F/M258L/S264A) ^g	61.7 \pm 4.7	0.6 \pm 0.04
X(I265S) ^h	25.1 \pm 2.6	1.2 \pm 0.1

^a Significantly different from N305R, $p < 0.0001$.

^b Not different from M1R, $p > 0.05$.

^c Significantly different from XY, $p < 0.0001$.

^d Significantly different from Y, $p < 0.05$, Mann-Whitney U test.

^e Not different from Y, $p > 0.05$, Mann-Whitney U test.

^f Significantly different from Y, $p < 0.01$.

^g Significantly different from XY, $p < 0.0001$.

^h Significantly different from X, $p < 0.001$.

ⁱ Extrapolated.

(the M1-R receptor). This chimeric receptor displayed higher DHEAS sensitivity, equivalent to the UNC-49B/C heteromer (Fig. 3, B and C; Table 1). Therefore, DHEAS and PS sensitivities are both increased by swapping in the UNC-49C M1 domain and restoring the conserved positive charge in the M2-3 linker, providing further evidence for a shared structural basis for the inhibition by the two neurosteroids.

DHEAS Sensitivity Depends on Some of the Same M1 Residues as PS Sensitivity. Within M1, PS sensitivity was increased by replacing a pair of divergent residues, N259 and V261, with their conserved counterparts from UNC-49C (Q and F, respectively; Fig. 4A) (Wardell et al., 2006). To test whether these same M1 residues control DHEAS sensitivity, we compared DHEAS dose-response curves from UNC-49B (N305R) homomers and from QF-R receptors, which contained the N259Q, V261F, and N305R mutations. The DHEAS sensitivity of QF-R was increased to the level of the M1-R receptor, significantly greater than the UNC-49B (N305R) receptor (Fig. 4B; Table 1). Therefore, the N259Q and V261F mutations seem to fully account for the ability of the UNC-49C M1 domain to increase DHEAS sensitivity. This result indicates that the conserved Q and F residues are important for both PS and DHEAS inhibition, demonstrating further similarity

between the two neurosteroids. However, this analysis also revealed a difference between the two neurosteroids. DHEAS was equally potent as an inhibitor of the QF-R and

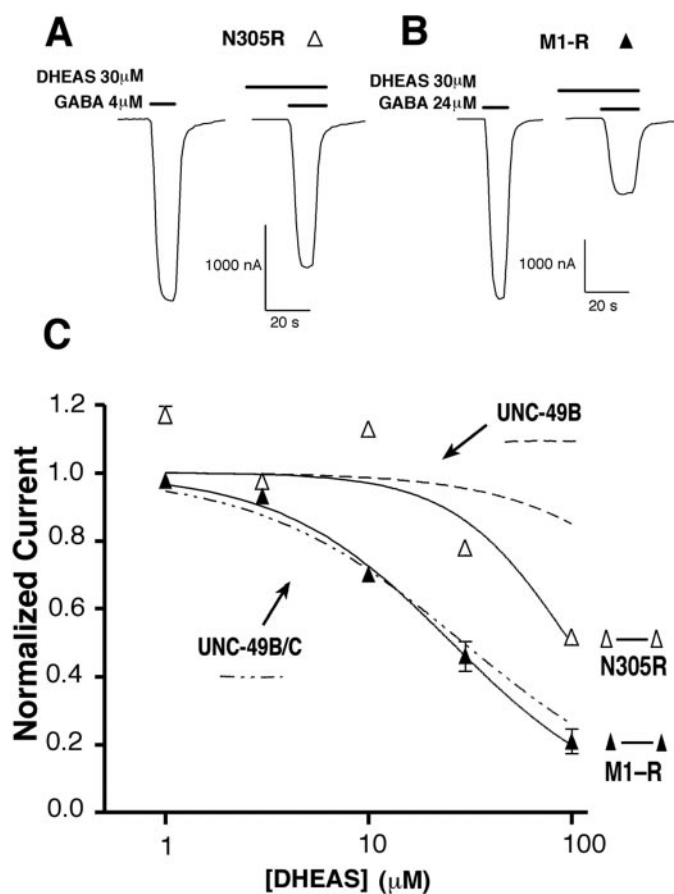


Fig. 3. UNC-49C M1 and the N305R mutation in the M2-3 linker increase DHEAS sensitivity. Representative traces showing inhibition of N305R receptors (A) and M1-R receptors (B) by 30 μ M DHEAS. DHEAS was preapplied for 20 s before coapplication of DHEAS and GABA. (C) DHEAS dose-response curves comparing N305R (open triangles) and M1-R (closed triangles; $n = 4$ for each receptor). DHEAS inhibition was measured at EC_{50} GABA. DHEAS dose-response curves for UNC-49B and UNC-49B/C receptor replotted from Fig. 2.

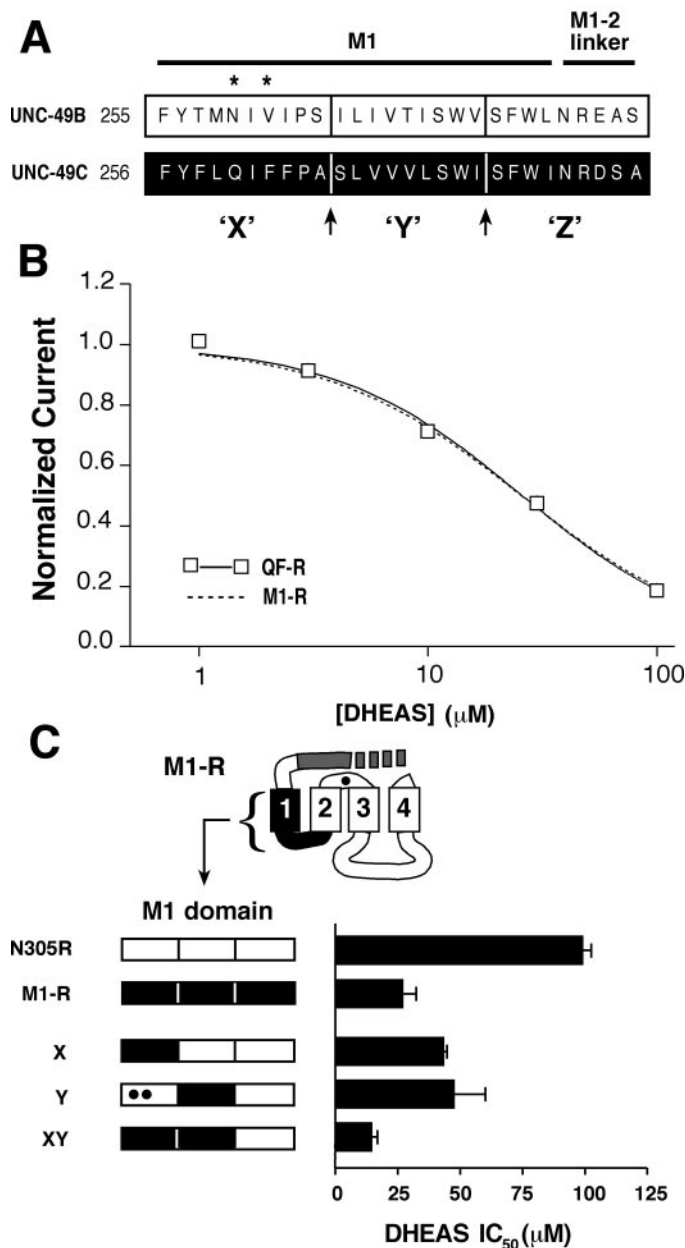


Fig. 4. M1 segments and residues that affect PS sensitivity also affect DHEAS sensitivity. A, subdivision of the M1 domain, including the M1-M2 linker, into three segments, designated X, Y, and Z. Asterisks indicate conserved residues at positions 259 and 261, arrows represent boundaries of the X segment (residues 255–264), Y segment (residues 265–273), and Z segment (residues 274–282). B, DHEAS dose response of the QF-R receptor, containing the N259Q, V261F, and N305R substitutions (open squares, solid line). The M1-R DHEAS dose-response from Fig. 3 is included for comparison (dashed line). C, DHEAS sensitivity of receptors with chimeric M1 segments containing different combinations of UNC-49B and UNC-49C X, Y, and Z segments. These receptors also contain the N305R mutation, and the chimera designated Y contains the N259Q and V261F mutations as well. Subunit diagrammed at top, with UNC-49C M1 segment shown in black. M1 plus M1-M2 linker region shown at left, indicating the combinations of UNC-49C and UNC-49B sequences tested. Black portions are UNC-49C, white portions are UNC-49B, and black dots represent UNC-49B to UNC-49C substitutions at positions 259, 261, and 305. DHEAS IC_{50} values were determined by generating DHEAS dose-response curves at EC_{50} GABA ($n = 4$ oocytes for each molecule; Table 1).

M1-R receptors, indicating that Q259 and F261 are principally important for DHEAS inhibition. PS, by contrast, is 9-fold more potent an inhibitor at the M1-R receptor compared with the QF-R receptor (Wardell et al., 2006), indicating that other M1 residues play major roles.

The remaining residues in M1 required for full PS sensitivity were demonstrated previously using a series of additional chimeras and M1 domain mutants (Wardell et al., 2006). We tested the DHEAS sensitivity of some of these mutants to determine whether further parallels between PS and DHEAS could be uncovered. Wardell et al. (2006) divided M1 into three segments of roughly equal length (X, Y, and Z; Fig. 4A). Maximal PS sensitivity was observed when UNC-49C X and Y segments were swapped together into UNC-49B, along with the N305R mutation, to generate a molecule called the XY chimera. Swapping the X or Y segments individually into UNC-49B N305R (the X and Y chimeras, respectively) produced submaximal PS sensitivity. We observed similar effects for DHEAS: the XY chimera showed the highest DHEAS sensitivity, significantly greater than either the X or the Y chimera (Fig. 4C; Table 1).

Analysis of individual residues further extended the parallels between the two steroids. First, mutating isoleucine 265 to serine in the X chimera significantly increased both PS and DHEAS sensitivity (Fig. 5A; Table 1). Second, mutating threonine 257 to phenylalanine in the Y chimera increased PS sensitivity, and it similarly increased DHEAS sensitivity (Fig. 5B, Table 1). Third, mutating phenylalanine 262 in the XY chimera to isoleucine reduced PS sensitivity and similarly reduced DHEAS sensitivity (Fig. 5B; Table 1). However, some mutations had different effects on the two steroids. Mutating serine 264 in the Y chimera had little effect on PS sensitivity but reduced DHEAS sensitivity 2- to 5-fold. Mutating methionine 258 to leucine produced biphasic PS dose-response curves with significant enhancement below 30 μ M. This mutation caused only small shifts in IC_{50} for DHEAS inhibition (Fig. 5B; Table 1), and enhancement was not observed (data not shown).

In summary, a parallel effect on DHEAS and PS inhibition was observed upon mutation of the pair of conserved residues (259 and 261) and three of the five other residues within M1 (257, 262, and 265). Differential effects were observed when two additional residues were mutated (residues 258 and 264; Table 2). Linear least-squares fit of PS sensitivity plotted against DHEAS sensitivity (Fig. 6) results in a line with a significantly nonzero slope ($p < 0.01$; $R^2 = 0.56$). Therefore, the change in DHEAS sensitivity correlates with the change in PS sensitivity among the mutant receptors tested. These data provide evidence that the mechanisms for DHEAS and PS inhibition of GABA_A receptors are similar. However they also demonstrate that substituting a carbonyl oxygen for an acetyl group at C17 directly or indirectly alters the way that a neurosteroid interacts with M1 domain residues.

UNC-49 Can Distinguish between PS Enantiomers but Not DHEAS Enantiomers. The conclusion from the above-mentioned experiments is not in agreement with earlier work that suggested that DHEAS and PS inhibit GABA_A receptor function by different mechanisms. Specifically, enantiomers of DHEAS had different inhibitory potencies at mammalian GABA_A receptors, whereas enantiomers of PS did not (Nilsson et al., 1998). The enantioselectivity of DHEAS implies that this compound inhibits

directly by binding a specific chiral recognition site on the receptor that causes allosteric inhibition. The lack of enantioselectivity for PS implies that it could act indirectly, by partitioning into and changing the physical properties of the lipid bilayer, altering the physical milieu of the receptor with functional consequences. To resolve this conundrum, we tested the enantiomers of PS and DHEAS on the *C. elegans* GABA_A receptor. We used a variant of UNC-49B called UNC-49B-PS7, containing seven mutations that increase PS sensitivity 58-fold (Wardell et al., 2006). UNC-49B-PS7 displayed enantioselectivity for PS; the IC_{50} for the unnatural enantiomer was 3-fold higher than for the natural enantiomer (Fig. 7A). We were surprised to find that UNC-49B-PS7 displayed no enantioselectivity for DHEAS. DHEAS and *ent*-DHEAS produced identical dose-response curves (Fig. 7B). This result is opposite to what was reported previously for mammalian

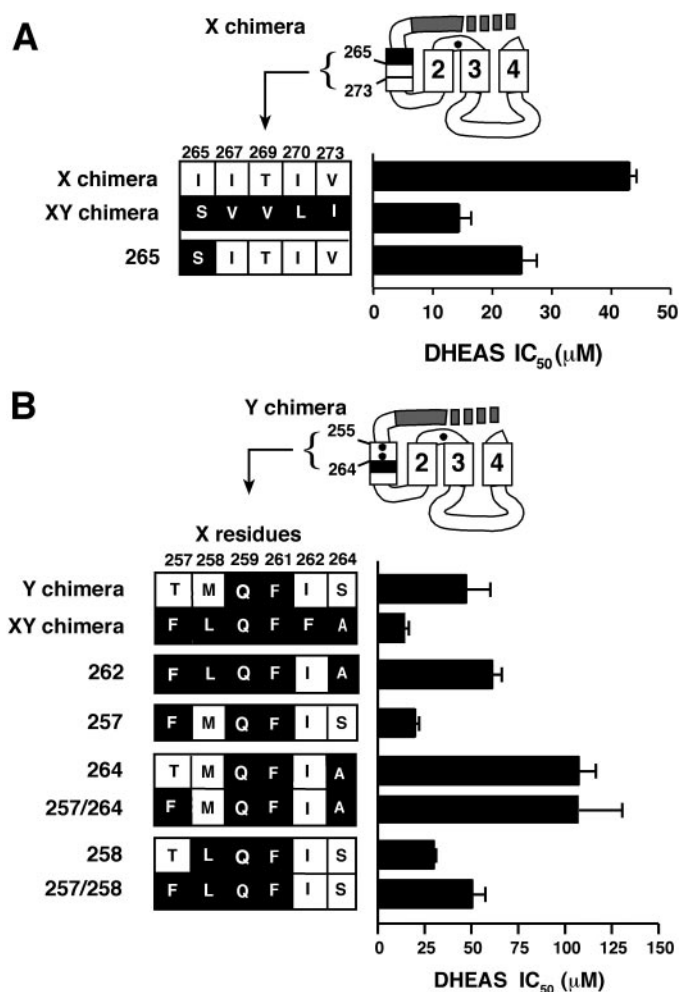


Fig. 5. Effects on DHEAS sensitivity of M1 point mutations known to affect PS sensitivity. A, residue 265 of the Y segment increases DHEAS sensitivity of the X chimera. Subunit diagrammed as in Fig. 3. Grid indicates Y segment residues that are different between UNC-49B and UNC-49C. X and XY DHEAS IC_{50} values are from Fig. 3. Black boxes and white letters are UNC-49C, and white boxes and black letters are UNC-49B. B, effects on DHEAS sensitivity of UNC-49B to UNC-49C substitutions within the X segment that are known to control PS sensitivity. Grid indicates residues that are different between UNC-49B and UNC-49C. Black dots on subunit diagrams represent UNC-49B to UNC-49C mutations at positions 259, 261, and 305. DHEAS IC_{50} values were determined by generating DHEAS dose-response curves at EC_{50} GABA ($n \geq 4$ for each molecule; Table 1).

GABA_A receptors (Nilsson et al., 1998). To confirm that the steroid preparations contained different DHEAS enantiomers, we demonstrated that they inhibited EC₅₀ GABA-evoked currents of mammalian $\alpha 1\beta 2\gamma 2L$ GABA_A receptors expressed in *X. laevis* oocytes with different potencies (data not shown). Therefore, enantioselectivity for sulfated neurosteroids is not consistent from one GABA_A receptor to another. We conclude that selectivity of GABA_A receptors for neurosteroid enantiomers is probably a function of the specific way that a particular steroid contacts its binding site on the receptor and that the absence of observed enantioselectivity does not unequivocally distinguish a direct binding mechanism from an indirect membrane-partitioning mechanism.

Discussion

The objective of this study was to compare the mechanisms by which the sulfated neurosteroids DHEAS and PS inhibit GABA_A receptors. These two steroids are identical except for the substituent at C17 of the steroid D ring, where DHEAS contains a carbonyl oxygen and PS contains an acetyl group. We examined the DHEAS inhibition of a series of wild-type and mutant variants of the *C. elegans* UNC-49 GABA_A receptor that were known from a previous study to have different sensitivity to PS inhibition. In general, we observed parallel effects with both neurosteroids, but several M1 mutations affected the inhibitory potency of the two neurosteroids differently.

Most receptor alterations had similar effects on the potencies of DHEAS and PS. First, DHEAS and PS sensitivities were higher in heteromers composed of UNC-49B and UNC-49C subunits compared with homomers composed of only UNC-49B subunits. Second, higher sensitivity to both steroids could be transferred to the UNC-49B subunit by swapping in UNC-49C sequences within the M1 domain and the M2-3 linker domain. Third, mutation of five M1 residues had parallel effects on DHEAS and PS sensitivities (summarized in Table 2; Fig. 6). Therefore, a major conclusion of this study is that the two neurosteroids have similar inhibitory mechanisms. This finding is not surprising, considering that the two neurosteroids have only one minor structural difference.

In contrast, several earlier studies have come to the conclu-

sion that PS and DHEAS inhibit GABA_A receptors through different mechanisms. First, at mammalian GABA_A receptors, the natural enantiomer of DHEAS inhibits with higher potency than the unnatural enantiomer, whereas for PS both enantiomers inhibit with equivalent potency (Nilsson et al., 1998). These results were interpreted to suggest that DHEAS interacts with a chiral recognition site on the GABA_A receptor, whereas PS might inhibit nonspecifically, by partitioning into the membrane, changing its physical properties and leading to reduced receptor function. We observed the opposite enantioselectivity profile with the UNC-49 receptor: DHEAS enantiomers have equivalent potencies, whereas PS enantiomers are different. Because drug mechanisms have generally proven to be conserved among the members of the ligand-gated ion channel family (Pribilla et al., 1992; Ffrench-Constant et al., 1993; Mihic et al., 1997; Etter et al., 1999; Pistis et al., 1999), it is unlikely that the UNC-49 receptor has completely different mechanisms of PS and DHEAS inhibition compared with its mammalian homologs. Instead, it is more likely that enantioselectivity is a function of the specific interatomic contacts that are made when a steroid binds to its binding site. Presumably the precise way that the two neurosteroids fit into their binding sites can explain why one receptor is enantioselective for a particular neurosteroid, whereas another is not.

Other data suggesting that DHEAS and PS might have different mechanisms come from radioligand binding studies. Specifically, PS inhibits binding of [³⁵S]*t*-butylbicyclopentylphosphorothionate and [³H](1-phenyl)-4-*t*-butyl-2,6,7-trioxabicyclooctane to rat brain membranes, whereas DHEAS does not (Majewska and Schwartz, 1987; Majewska et al., 1990; Demirgören et al., 1991). These studies were interpreted to indicate that PS and DHEAS bind to different binding sites on the GABA_A receptor. However, these data cannot rule out the possibility that the two steroids have very similar binding sites and transduction mechanisms, but the small structural difference between the steroids exerts enough of a steric effect to cause measurable differences in the binding of radiolabeled probes to other sites on the receptor. Taken together, our mutation data and data from these earlier studies are best explained with a model in which PS and DHEAS both bind directly to the GABA_A receptor and activate a downstream transduction pathway that leads to receptor

TABLE 2

Comparison of the effects of receptor structural changes on DHEAS and PS sensitivity

Bold rows indicate where modifications have nonparallel effects on the two steroids.

Modification	Effect on Dheas Sensitivity	Effect on PS Sensitivity
Addition of UNC-49C subunit	17.1-fold ↑ ^a	83.0-fold ↑ ^a
N305R mutation	5.1-fold ↑ ^a	3.7-fold ↑ ^a
N305R mutation plus UNC-49C M1 domain	18.4-fold ↑ ^a	68.2-fold ↑ ^a
N259Q/V261F/N305R triple mutation	19.2-fold ↑ ^a	7.7-fold ↑ ^a
Replace M1 X segment with UNC-49C X residues	2.3-fold ↑ ^b	4.0-fold ↑ ^b
Replace M1 Y segment with UNC-49C Y residues	2.1-fold ↑^b	1.8-fold ↓^b
Replace M1 XY segment with UNC-49C XY residues	6.8-fold ↑ ^b	13.8-fold ↑ ^b
I265S mutation	1.7-fold ↑ ^c	3.7-fold ↑ ^c
T257F mutation	2.4-fold ↑ ^d	4.9-fold ↑ ^d
M258L mutation	1.6-fold ↑^d	Biphasic response^d
S264A mutation	2.3-fold ↓^d	No significant change^d
F262I mutation	4.2-fold ↓ ^e	12.0-fold ↓ ^e

↑, increase; ↓, decrease.

^a Compared with wild-type UNC-49B.

^b Compared with N305R mutant.

^c Compared with X chimera.

^d Compared with Y chimera.

^e Compared with XY chimera.

inhibition. Although not identical, the binding and transduction mechanisms probably involve many of the same receptor residues.

Although most receptor mutations had parallel effects on PS and DHEAS, several alterations within the M1 domain had differential effects on the inhibition of UNC-49 by the two neurosteroids. First, the S264A mutation had little effect on PS, but it led to a 2- to 5-fold reduction of DHEAS potency. Second, the M258L mutation had little effect on DHEAS, but it unmasked an enhancing activity of PS at certain concentrations. Third, although the QF-R receptor is equally sensitive to PS and DHEAS, the M1-R receptor shows 9-fold higher sensitivity for PS but unchanged sensitivity for DHEAS. Therefore, M1 residues other than Q259 and F261 are important for discriminating between PS and DHEAS. Considering that the two steroids differ only by the identity of the substituent at C17 on the D ring, the most straightforward interpretation of these differential effects is that the steroid D ring contacts the M1 domain. This interpretation is based on the simplifying principle that perturbations are likely to have their strongest steric and chemical effects locally: the strongest effects of a mutation are likely to be felt in the local environment of the altered residue, and the strongest effects of a structural alteration to a ligand are likely to be felt where the altered moiety contacts the receptor. Therefore, to observe that mutating an M1 residue differentially affects PS and DHEAS inhibition suggests that the C17 substituent makes contact with M1 residues, that those residues directly or indirectly accommodate the C17 substituent upon binding, and that those residues play different transduction roles for the two steroids, accounting for the differential effects of mutation. We observed three separate examples of alterations in M1 sequence causing differential effects on PS and DHEAS, supporting the idea that the D ring contacts M1. An alternative explanation that we cannot rule out is that the D ring binds a different part of the

receptor, and mutations in M1 and substitution at C17 of the steroid have their effects over a longer distance. However, neurosteroid interactions with the GABA_A receptor seem to be generally tolerant of significant modification of the steroid backbone that might be expected to affect the long-range propagation of steric binding effects. These include alteration of the stereochemistry of the 5-carbon, and opening of the A ring, resulting in a more-flexible benz[e]indene steroid analog (Akk et al., 2007). Those findings suggest that local effects of a mutation or steroid structural alteration are probably more important than long-range changes propagating from one end of the steroid binding pocket to the other.

The possibility that DHEAS and PS bind the GABA_A receptor with the D ring contacting the M1 domain is significant because pregnane neurosteroids that enhance GABA_A receptor function, such as pregnanolone, are thought to bind the mammalian GABA_A receptor $\alpha 1$ subunit in the opposite

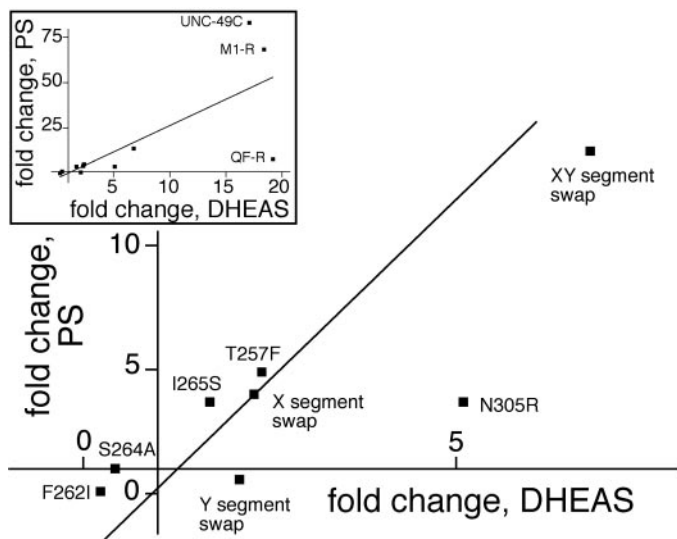


Fig. 6. Parallel effects of receptor mutation on PS and DHEAS sensitivities. Scatter plot showing the correlation between the relative changes in DHEAS IC_{50} (x-axis) and PS IC_{50} (y-axis) that result from mutating the receptor (each data point is labeled with the corresponding mutation; see Table 2). Mutation of the M258 residue is not included in the plot. Intersection of axes represents wild-type sensitivity to both steroids (i.e., $X = 1$, $Y = 1$). Plot with expanded range is shown in the inset, to include mutants with largest shifts. Line is linear least-squares fit.

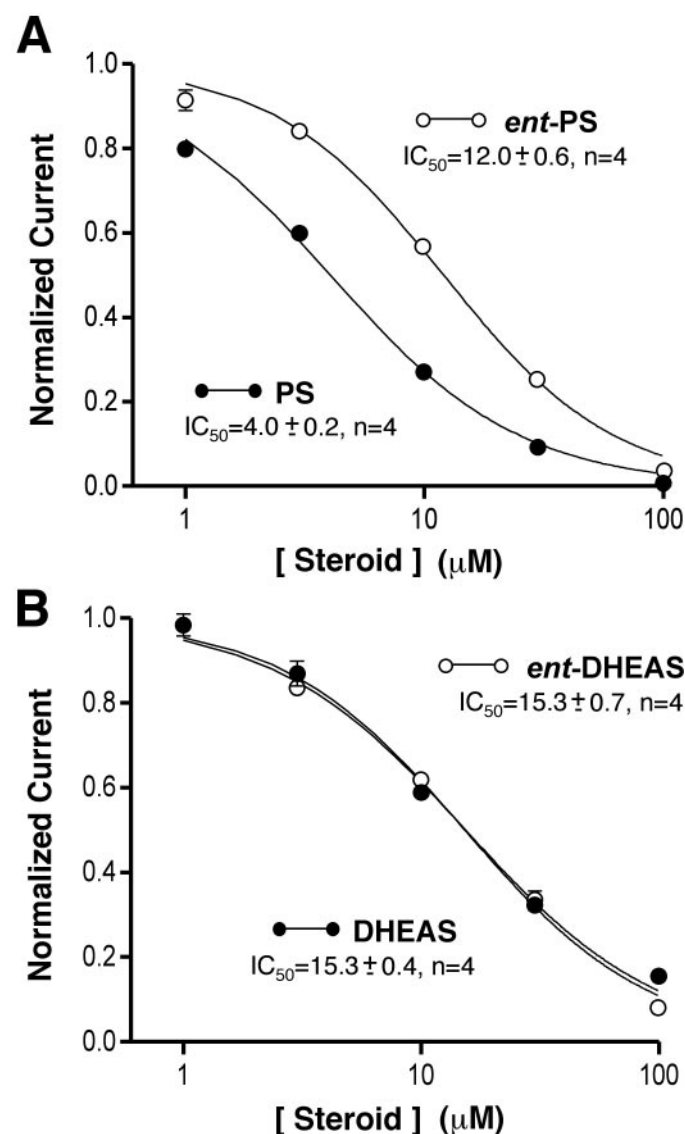


Fig. 7. UNC-49 receptor is enantioselective for PS but not DHEAS. A, PS and *ent*-PS dose-response curves of the UNC-49B-PS7 receptor (closed and open circles, respectively). B, DHEAS and *ent*-DHEAS dose-response curves of the UNC-49B-PS7 receptor (closed and open circles, respectively). All dose-response curves were measured at EC_{50} GABA ($n = 4$ oocytes for each steroid).

orientation. In this model, a hydroxyl group attached to C3 of the steroid A ring binds a hydrogen bond acceptor residue in the M1 domain, and the C17 substituent on the steroid D ring binds a hydrogen bond donor residue in the M4 domain (Hosie et al., 2006). It will be interesting to compare the structure-function requirements of enhancing and inhibitory neurosteroids at a single receptor type to determine whether the two kinds of steroids fit into the same or homologous binding pockets in opposite orientations. A better understanding of the diversity of neurosteroid modulatory mechanisms will be valuable for the design of novel neurosteroid site ligands that can be developed into therapeutic drugs.

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