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RESEARCH PAPER

Structurally diverse amphiphiles exhibit biphasic modulation of $GABA_A$ receptors: similarities and differences with neurosteroid actions

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Background and purpose: Some neurosteroids, notably 3α-hydroxysteroids, positively modulate $GABA_A$ receptors, but sulphated steroids negatively modulate these receptors. Recently, other lipophilic amphiphiles have been suggested to positively modulate GABA receptors. We examined whether there was similarity among the actions of these agents and the mechanisms of neurosteroids. Significant similarity would affect theories about the specificity of steroid actions.
Experimental approach: *Xenopus laevis* oocytes were challenged with Triton X-100, octyl-β-glucoside, capsaicin, docosahexaenoic acid and sodium dodecyl sulphate (SDS), along with different GABA concentrations.
Key results: These compounds have both positive and negative effects on GABA currents, which can be accentuated according to the degree of receptor activation. A low GABA concentration (1 μM) promoted potentiation and a high concentration (20 μM) promoted inhibition of current, except for SDS that inhibited function even at low GABA concentrations. Amphiphile inhibition was characterized by enhanced apparent desensitization and by weak voltage dependence, similar to pregnenolone sulphate antagonism. We then tested amphiphile effects on mutated receptor subunits that are insensitive to negative (α1V256S) and positive (α1Q241L or α1N407A/Y410F) steroid modulation. Negative regulation by amphiphiles was nearly abolished in α1V256S-mutated receptors, but potentiation was unaffected. In α1Q241L- or α1N407A/Y410F-mutated receptors, potentiation by amphiphiles remained intact.
Conclusions and implications: Structurally diverse amphiphiles have antagonist actions at $GABA_A$ receptors very similar to those of sulphated neurosteroids, while the potentiating mechanisms of these amphiphiles are distinct from those of neurosteroid-positive modulators. Thus, such antagonism at $GABA_A$ receptors does not have a clear pharmacophore requirement.
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Abbreviations: 3α5αP, (3α,5α)-3-hydroxypregnan-20-one; DHA, docosahexaenoic acid; OG, octyl-β-glucoside; PS, pregnenolone sulphate; SDS, sodium dodecyl sulphate; WT, wild-type

Introduction

Fast inhibitory neurotransmission in the CNS is largely mediated by chloride-permeable ion channels activated by GABA ($GABA_A$ receptors; nomenclature follows Alexander *et al.*, 2009) or glycine. $GABA_A$ receptor function is potently modulated by several agents including neuroactive steroids, which can be synthesized *de novo* in the brain or can enter the brain from the periphery (Baulieu *et al.*, 2001; Belelli and Lambert, 2005). Natural and synthetic analogues of 3α-hydroxysteroids are anaesthetics, anticonvulsants, sedatives and anxiolytics (Gasior *et al.*, 1999; Zorumski *et al.*, 2000) probably because they directly enhance the actions of GABA. By contrast, endogenous sulphated steroids such as pregnenolone sulphate (PS) and various sulphated pregnane steroids potently inhibit $GABA_A$ receptors (Majewska *et al.*, 1988; Akk *et al.*, 2001; Eisenman *et al.*, 2003) through a non-competitive mechanism. Although positive and negative steroid modulators have potent, direct actions at $GABA_A$ receptors, the inherent affinity of neurosteroids for the receptor sites may be quite low, with potency resulting from the strong lipophilicity of steroids and the associated cellular retention (Akk *et al.*, 2005;

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Chisari *et al*., 2009). Based on this observation, it is possible that steroids act on GABA receptors via low-affinity sites that may accommodate other structures. If this hypothesis is confirmed, the structure–activity relationship for steroid-like actions may be much broader than previously realized. In the case of antagonist steroids, effects sometimes exhibit weak enantioselectivity (Nilsson *et al*., 1998) and lack of clear structure–activity requirements (Mennerick *et al*., 2001). Therefore, it is possible that antagonism lacks a specific binding site.

Recently, several amphiphilic compounds were identified as $GABA_A$ receptor potentiators (Sogaard *et al*., 2006; Yang and Sonner, 2008). These compounds are structurally distinct from steroids, but among themselves share a common feature: a long hydrocarbon tail, which provides lipid solubility, and a hydrophilic head (either ionic or uncharged), that allows solubility in polar solvents. To investigate the hypothesis that these amphiphiles have mechanistically similar actions to neurosteroids, we used Triton X-100, octyl-β-glucoside (OG), capsaicin, docosahexaenoic acid (DHA) and sodium dodecyl sulphate (SDS). Figure S1 shows the structures of these compounds and two comparator neurosteroids. These detergents were selected because of previously published suggestions of interactions with $GABA_A$ receptors (Sogaard *et al*., 2006; Yang and Sonner, 2008) and to explore the possibility that anionic (SDS and DHA) and uncharged polar head groups with various length and degree of saturation in the hydrophobic chain can differently modulate $GABA_A$ receptor response.

To our surprise, we found that several amphiphiles exhibited a complicated mixture of potentiation and inhibition of GABA responses. Our data indicated that structurally diverse amphiphiles antagonized $GABA_A$ receptors and shared a similar mechanism with sulphated neurosteroids, while the potentiating mechanisms of these amphiphiles were distinct from those of neurosteroid-positive modulators. Although there is evidence for specific effects of sulphated neurosteroids at some GABA receptor classes (Li *et al*., 2006; Twede *et al*., 2007), our results were consistent with the idea that non-specific effects, possibly including membrane alterations that can be induced by structurally diverse amphiphiles, may underlie the antagonist actions of sulphated neurosteroids at $GABA_A$ receptors.

Methods

Oocyte expression studies

All animal care and experimental protocols were approved by the Washington University Animal Studies Committee. Stage V–VI oocytes were harvested from sexually mature female *Xenopus laevis* (Xenopus One, Northland, MI, USA) under 0.1% 3-aminobenzoic acid ethyl ester anaesthesia, according to protocols approved by the Washington University Animal studies Committee. Oocytes were defolliculated by shaking for 20 min at 37°C in collagenase (2 $mg \cdot mL^{-1}$) dissolved in calcium-free solution containing (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$ and 5 HEPES at pH 7.4. Capped mRNA, encoding rat $GABA_A$ receptor α1, β2 and γ2L wild-type (WT) subunits and α1V256S-, α1Q241L- and α1N407A/Y410F-mutated subunits was transcribed *in vitro* using the mMESSAGE mMachine Kit (Ambion, Austin, TX, USA) from linearized pBluescript vectors containing receptor coding regions. Subunit transcripts were injected in equal parts (20–40 ng total RNA) 16–24 h following defolliculation. Oocytes were incubated for up to 5 days at 18°C in ND96 medium containing (in mM): 96 NaCl, 1 KCl, 1 $MgCl_2$, 2 $CaCl_2$ and 10 HEPES at pH 7.4, supplemented with pyruvate (5 mM), penicillin (100 $U \cdot mL^{-1}$), streptomycin (100 $\mu g \cdot mL^{-1}$) and gentamycin (50 $\mu g \cdot mL^{-1}$).

Oocyte electrophysiology

Two-electrode voltage-clamp experiments were performed with a Warner OC725 amplifier (Warner Instruments, Hamden, CT, USA) 2–5 days following RNA injection. The extracellular recording solution was ND96 medium with no supplements. Intracellular recording pipettes were filled with 3 M KCl and had open tip resistances of ~1 MΩ. GABA and modulators were applied from a common tip via a gravity-driven multibarrel delivery system. Cells were voltage-clamped at −70 mV or at +50 mV as indicated. The peak current and the current at the last point of drug application were measured for quantification of current amplitudes.

Experimental procedures

Experiments in Figures 1, 2 and 4–7 were performed using the protocol shown in Figure 1A. Amphiphiles were pre-applied for 5 s before simultaneous application with GABA. After amphiphile treatment, oocytes were kept in saline for 40–50 s and challenged again with GABA alone to test current recovery. At least partial recovery occurred in all tested oocytes and is shown in the figures.

Data analysis and statistical procedures

Data acquisition and analysis were performed with pCLAMP 9.0 software (Molecular Devices, Union City, CA, USA). Data plotting and curve fitting were performed with Prism 5.01 software (GraphPad, San Diego, CA, USA). Data are presented in the text and figures as mean ± SE. Values were determined by measuring current at the peak and at the end of drug co-application, as indicated in results and figure legends. For GABA applications before and after amphiphiles, values were averaged, and this average baseline response is indicated with dotted lines in bar diagrams. Also, both potentiation and inhibition by amphiphiles are normalized to the averaged value of GABA applications before and after (recovery) modulator treatment. This analysis assumes that any partial recovery resulted from a linear, time-dependent rundown of GABA currents. If the partial recovery results from a permanent effect of modulator, some of our analyses may underestimate the true modulatory effect. Curve fitting for Figure 1D and E was performed on normalized values calculated as M/G, where M is the response in the presence of GABA + modulator and G is the response to GABA alone. In Figures 3 and S2 concentration–response curves were normalized to the highest GABA concentration applied to facilitate comparison of shapes and EC_{50} values. For each case, more details are given in figure legends. Empirical fits to concentration–

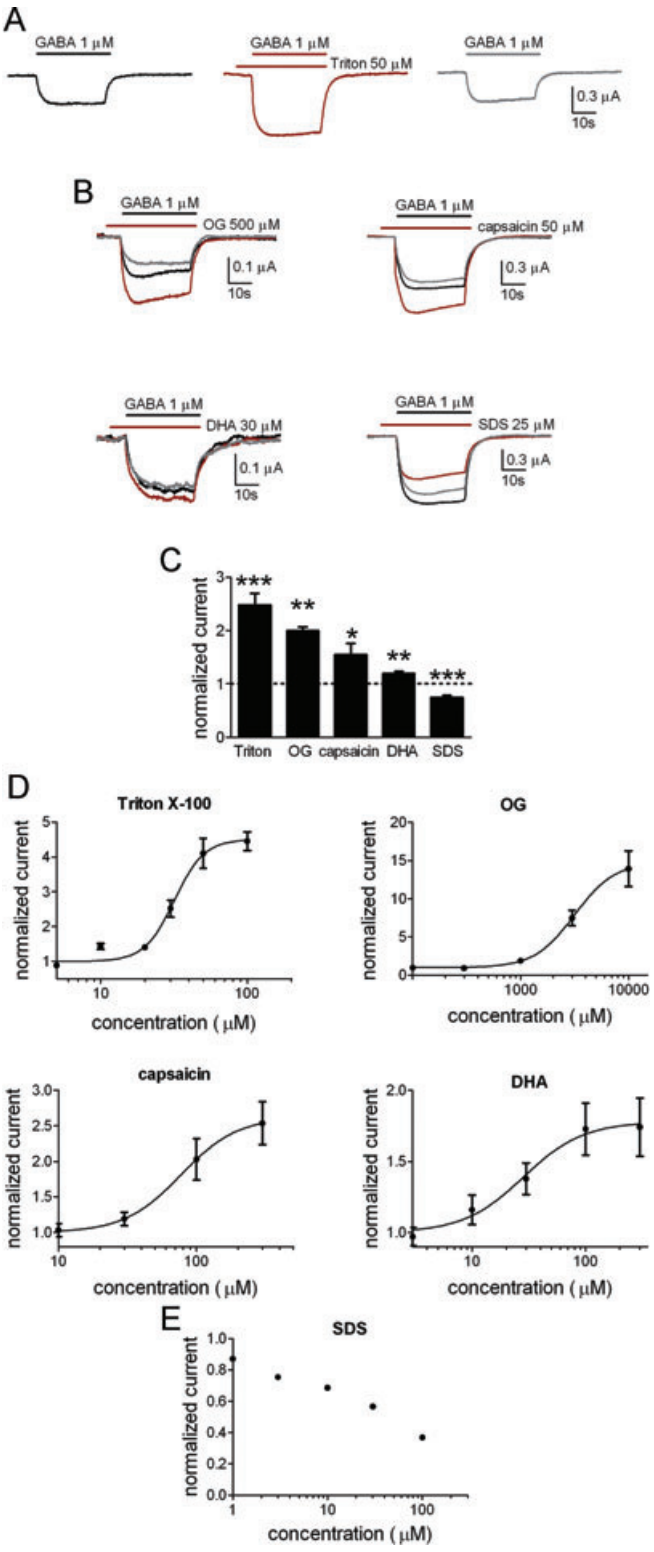

Figure 1 GABA current is typically potentiated by amphiphiles at low GABA concentration. (A) Representative traces of oocytes stimulated by 1 μM GABA (black trace, left) and by co-application of 50 μM Triton X-100 (red trace, middle). After Triton washout, GABA was reapplied to measure current recovery (grey trace, right). (B) Representative traces of other tested amphiphiles (as indicated). (C) Summary of peak current in oocytes treated as in (A and B). GABA responses were averaged (dotted line). Values are means ± SE [Triton, n = 8; octyl-β-glucoside (OG), n = 9, capsaicin, n = 10; docosahexaenoic acid (DHA), n = 10; sodium dodecyl sulphate (SDS), n = 7], $^*P < 0.05$, $^{**}P < 0.01$ $^{***}P < 0.0001$ (paired t-test), significantly different from responses to GABA alone (dotted line). (D) Increasing concentrations of Triton (10–300 μM, n = 7), OG (0.1–10 mM, n = 3), capsaicin (10–300 μM, n = 4) and DHA (3–300 μM, n = 6) were applied to *Xenopus laevis* oocytes expressing rat α1β2γ2L $GABA_A$ receptor subunits. Concentration–response curves were obtained in the presence of 1 μM GABA, and potentiation was expressed relative to the GABA response (expressed as 1) in the absence of amphiphiles. For display purposes, the lines represent fits of the Hill equation (adjusted to account for the lower asymptote of 1.0) to the averaged data points shown in each graph. Parameters from the summary fits for Triton, OG, capsaicin and DHA, respectively, were: EC_{50} 32 μM, 3160 μM, 77 μM and 28 μM; Hill coefficient: 7.7, 2.4, 2.1 and 1.6. (E) Increasing concentrations of SDS (1–100 μM, n = 5) on α1β2γ2L $GABA_A$ receptors expressed in *X. laevis* oocytes. This concentration–response curve was obtained in the presence of 20 μM GABA, and inhibition was expressed relative to the GABA response (treated as 1) in the absence of SDS. Limiting factors that prevented the use higher concentrations of amphiphiles were solution saturation (for OG and capsaicin) and plasma membrane damage (for Triton X-100 and SDS).

response relationships were achieved using a least-squares minimization to the Hill equation: $B_{max}[X^h/(EC_{50}{}^h + X^h)]$ where B_{max} is the maximum potentiation, h is the Hill coefficient, EC_{50} is the concentration of amphiphiles producing 50% of maximum potentiation, and X is the tested concentration. For display, solid lines are Hill equation fits to the averaged experimental data. Statistical differences were determined using Student's two-tailed t-test.

Throughout the work, we used low and high concentrations of GABA to evaluate potentiation and inhibition respectively. Based on inspection of the concentration–response relationships shown in Figure S2B and in previous work (Wang *et al.*, 2002), for low GABA, we chose 1 μM, 0.05 μM, 10 μM and 3 μM for WT, α1V256S, α1Q241L and α1N407A/Y410F respectively. In all cases, responses were below 6% of maximum. Therefore, these concentrations gave us comparable baseline GABA responses among the mutated receptors and allowed clear detection of potentiation. For high GABA concentration, we used 20 μM, 5 μM and 50 μM for WT, α1V256S and α1Q241L respectively. For the WT and α1Q241L receptors these concentrations represented EC 68 ± 4% and 66 ± 4%, respectively, from fits to individual oocytes. For the V256S mutation, we used a concentration closer to maximum (96 ± 4%) to help ensure detection of use-dependent antagonism.

Materials

All drugs were from Sigma (St. Louis, MO, USA) except for SDS that was from Life Science (Hercules, CA, USA).

Results

$GABA_A$ receptor modulation by amphiphiles

We tested amphiphiles in *X. laevis* oocytes injected with RNA coding for $GABA_A$ receptors (α1β2γ2L). For reference, structures of amphiphiles tested in this work are shown in Fig-

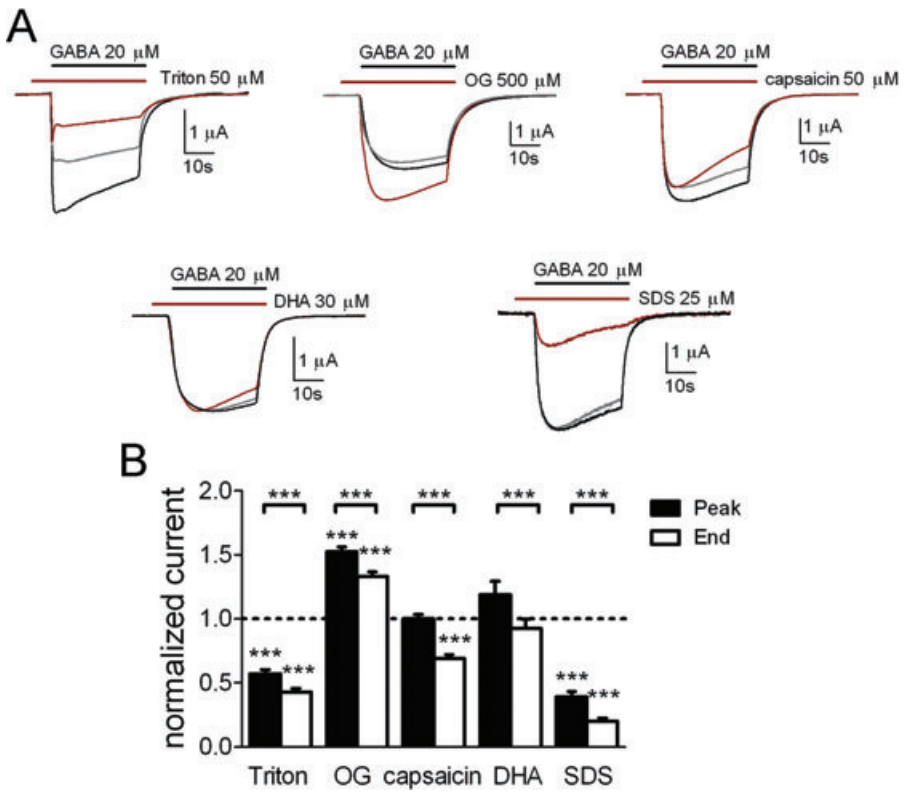

Figure 2 GABA current is inhibited by amphiphiles at high GABA concentration. (A) Representative traces of oocytes stimulated by 20 μM GABA (black trace) and by co-application of amphiphiles (red traces, as indicated). GABA was reapplied after each modulator to measure current recovery (grey traces). (B) Summary of normalized current (peak and end of drug application) in oocytes treated as in (A). Dotted horizontal line represents averaged GABA responses before and after drug application. Values are means ± SE [Triton, $n = 14$; sodium dodecyl sulphate (SDS), $n = 6$; octyl-β-glucoside (OG), $n = 10$; capsaicin, $n = 16$; docosahexaenoic acid (DHA), $n = 16$], ***$P < 0.0001$ (paired *t*-test), significantly different from responses to GABA alone (dotted line).

ure S1. The protocol for our screen of amphiphile effects is shown in Figure 1A. Initially, we applied a low GABA concentration, 1 μM, which should yield a response that is ~3% of maximum (EC_{50} ~10 μM) in oocytes expressing the α1β2γ2L subunit combination (Eisenman *et al.*, 2003). Amphiphiles were applied for 5 s before co-application with GABA. Pre-applications demonstrated only very small or no direct currents to amphiphiles at the tested concentrations (Figure 1). A subsequent application of GABA alone was used to measure current recovery. Amphiphile effects exhibited at least partial recovery (Figure 1A and B). In some cases underlying rundown of GABA responses was evident, but overall reversibility indicated that the concentrations used for each compound did not permanently modify membrane properties or GABA receptor function. For Triton X-100 (50 μM), OG (500 μM), capsaicin (50 μM) and DHA (30 μM) we observed potentiation of GABA currents (Figure 1A and B), but SDS (25 μM) inhibited currents (Figure 1B). The extent of potentiation and inhibition of GABA current by amphiphiles at the indicated concentrations is shown in Figure 1C.

Concentrations of Triton X-100, OG, capsaicin and DHA used here were based on half maximal effective concentrations (EC_{50}) estimated from concentration–response curves in presence of 1 μM GABA (Figure 1D). Concentration ranges for each compound were: Triton X-100, 0.01–0.3 mM; OG, 0.1–10 mM; capsaicin 0.01–0.3 mM; DHA, 0.003–0.3 mM. For SDS (0.001–0.1 mM), which exhibited only inhibition, concentration–response profiles of the half maximal inhibitory concentration (IC_{50}) were determined in presence of 20 μM GABA, a GABA concentration at which we have previously observed reliable and robust inhibition by sulphated and 3β-hydroxysteroids (Wang *et al.*, 2002; Eisenman *et al.*, 2003) (Figure 1E). As will be shown below, the actions of amphiphiles suggest a complicated mixture of positive and negative modulation. Thus, our measures of both effective concentrations and maximum modulation are likely to be confounded by the presence of mixed positive and negative modulation. Evaluation of the half-maximum concentrations and the maximum predicted potentiation by amphiphiles on GABA current suggests that amphiphiles are less potent and less efficacious (except possibly in the case of OG) than the neurosteroid (3α,5α)-3-hydroxypregnan-20-one (3α5αP) in potentiating GABA current (Figure 1D). Typical potency and efficacy for 3α5αP in this assay is 1 μM EC_{50} and 10–20 fold maximum potentiation (Wang *et al.*, 2002; Chisari *et al.*, 2009).

The degree of potentiation and inhibition by neuroactive steroids can be influenced by GABA concentration. For instance, the inhibition by sulphated steroids increases with receptor activation (Eisenman *et al.*, 2003). Therefore, we evaluated the effect of amphiphiles on GABA current in presence of a high agonist concentration, 20 μM, which should

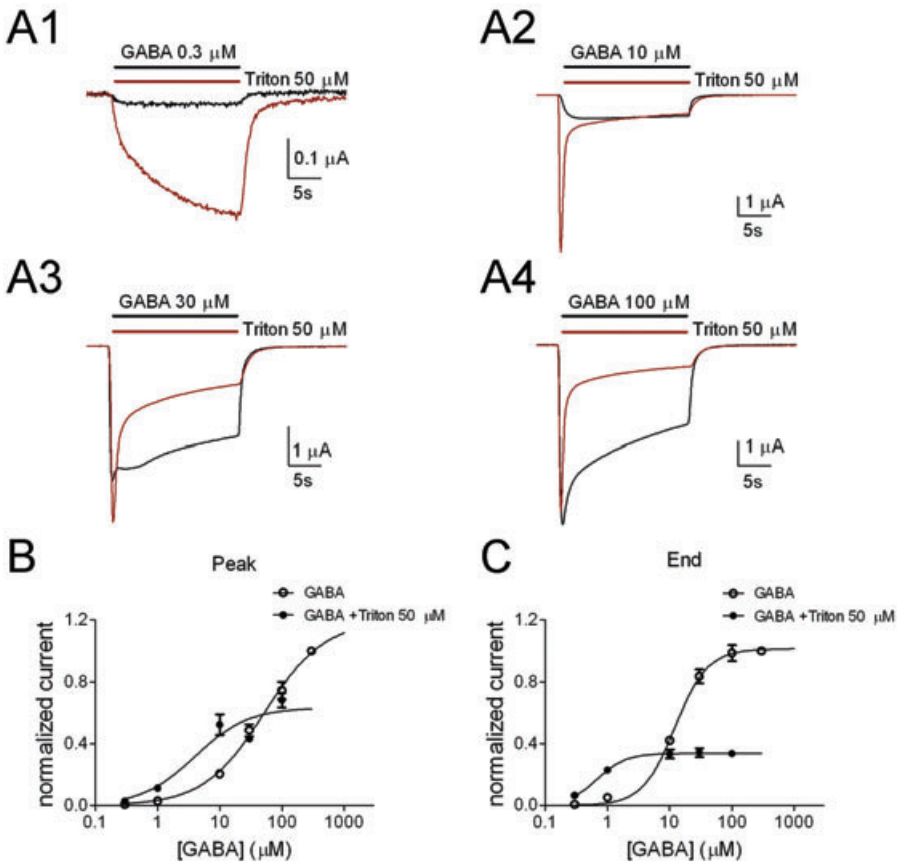

Figure 3 The effects of Triton X-100 at multiple GABA concentrations. (A1–A4) Representative responses from one oocyte to increasing GABA concentrations (0.3–100 μM) in the presence and absence (as indicated) of 50 μM Triton X-100. For these experiments GABA and Triton X-100 were simultaneously co-applied; there was no pre-application of modulator. (B and C) Concentration–response curves of current at the peak and at the end of drug application (as indicated) resulting from increasing GABA concentrations alone (0.3–300 μM) and in the presence of 50 μM Triton X-100 (n = 10–14). Responses were expressed relative to that of the highest GABA concentration applied (300 μM). For display purposes, the lines represent fits of the Hill equation to the averaged data points shown in the figure. Note that because of the mixture of effects, a single Hill equation is likely not an appropriate model, so the parameters from these fits are of empirical value only. Parameters from the summary fits for GABA alone and in presence of Triton X-100, respectively, were: for the peak, EC_{50} 52.2 μM and 2.7 μM, Hill coefficient 0.9 and 1.7; for the end, EC_{50} 12.4 μM and 0.7 μM, Hill coefficient 1.7 and 1.8.

Table 1 Summary of amphiphile effects in WT and mutated $GABA_A$ receptors

	αWT		*α1V256S*		*α1Q241L*		*αN408A/Y411F*
GABA (μM)	1	20	0.05	5	10	50	3
Triton X-100	2.47 ± 0.22	0.57 ± 0.04	6.48 ± 0.39***	1.16 ± 0.04***	1.67 ± 0.12*	ND	1.88 ± 0.19
OG	1.99 ± 0.08	1.53 ± 0.04	2.41 ± 0.14*	1.20 ± 0.01***	1.93 ± 0.03	ND	1.57 ± 0.11**
capsaicin	1.55 ± 0.21	1.00 ± 0.03	2.19 ± 0.14	1.02 ± 0.04	1.71 ± 0.04	ND	1.60 ± 0.13
DHA	1.19 ± 0.04	1.19 ± 0.11	1.67 ± 0.14**	0.99 ± 0.01	1.28 ± 0.03	ND	1.36 ± 0.09
SDS	0.75 ± 0.04	0.39 ± 0.04	1.21 ± 0.08***	0.84 ± 0.02***	ND	0.53 ± 0.05	ND

Means ± SE indicate peak current of amphiphiles traces normalized to GABA applications (average of the first application and recovery, treated as 1). Concentrations for amphiphiles were Triton X-100, 50 μM; OG, 500 μM; capsaicin 50 μM; DHA, 30 μM; SDS, 25 μM. For N408A/Y411F mutation: Triton X-100, n = 5; OG, n = 6; capsaicin, n = 6; DHA, n = 5.

*P < 0.05, **P < 0.01, ***P < 0.0001 (unpaired t-test) show significant differences in mutated receptors compared with WT in the corresponding GABA activated state.

DHA, docosahexaenoic acid; ND, not determined; OG, octyl-β-glucoside; SDS, sodium dodecyl sulphate; WT, wild-type.

yield a response that is ~70% of maximum in oocytes injected with α1β2γ2L $GABA_A$ receptors (Eisenman *et al.*, 2003). As shown in Figure 2A, for SDS and OG the direction of modulation was the same as that observed with low GABA concentration (inhibition for SDS and potentiation for OG). Quantitatively, however, OG potentiation was decreased and SDS inhibition was increased (Table 1).

For the other three compounds tested, the direction of modulation reversed at high GABA concentration, and net inhibition was observed. Triton X-100 exhibited a strong inhibition in presence of 20 μM GABA. This inhibition was characterized by a rapidly decaying response upon application of GABA (Figure 2A). The kinetics were faster than for the other compounds, although the overall pattern of increased appar-

ent desensitization was typical of all compounds examined. For capsaicin and DHA, peak responses with 20 μM GABA were not significantly altered, but apparent desensitization increased. For all amphiphiles tested, significant inhibition developed by the end of the co-application of modulator with GABA, resulting in apparently enhanced desensitization of GABA current (Figure 2A and B). This effect largely recovered upon wash out of modulator (Figure 2A), suggesting a largely acute, reversible effect of these compounds.

To analyse more clearly the mixed potentiation/inhibition effects of Triton X-100 on $GABA_A$ receptors, full concentration–response curves with a fixed Triton X-100 concentration (50 μM) and increasing GABA concentrations (0.3–100 μM) were obtained (Figure 3). In these experiments GABA and the modulator were co-applied with no pre-application period. Currents at both peak and the end of drug application were evaluated, and current was normalized to the response to 300 μM GABA in the absence of Triton X-100. As expected, in the presence of low GABA concentrations (0.3 and 1 μM) Triton X-100 exhibited strong potentiation of both peak and final currents (Figure 3A1, B and C). At 10 μM GABA, the peak current showed strong potentiation in the presence of Triton X-100, but at the end of drug application strong desensitization/inhibition was observed. Peak currents decayed very quickly with this co-application protocol, and peak amplitude potentiation varied substantially from oocyte to oocyte at the intermediate GABA concentrations (10 and 30 μM). Because of inherently slow drug delivery to large oocytes and because of the dynamic mixture of potentiation and inhibition of GABA responses, it seems likely that there is considerable error in the estimates of peak amplitudes (Figure 3B), although qualitatively it was clear that at higher concentrations of GABA, potentiation decreased (Figure 3A4 and B). Responses at the end of GABA applications were more easily interpretable because they were less contaminated by the dynamics of solution exchange on the oocytes (Figure 3A1–A4 and C). Here, the evidence of potentiation at low GABA concentrations and the inhibition at high GABA concentrations was clear (Figure 3C).

Superficially, the enhanced desensitization by amphiphiles shares similarity with sulphated steroid and picrotoxin antagonism of GABA responses, with the degree of antagonism depending upon receptor activation (Inoue and Akaike, 1988; Yoon *et al.*, 1993; Eisenman *et al.*, 2003). PS has no detectable voltage dependence, and sulphated pregnane steroids exhibit weak voltage dependence (Akk *et al.*, 2001; Mennerick *et al.*, 2001; Eisenman *et al.*, 2003). Picrotoxin antagonism also exhibits weak, if any voltage dependence (Newland and Cull-Candy, 1992). We tested the voltage dependence of inhibition by the two strongest negative modulators, Triton X-100 and SDS.

Previous studies have shown that in presence of 100 μM GABA, the GABA current/voltage relationship is almost linear (Mennerick *et al.*, 2001; Pytel *et al.*, 2005). Therefore, we used an elevated GABA concentration for these experiments. This reduced the possibility of confusing voltage-dependent receptor gating with voltage-dependent drug effects (Mennerick *et al.*, 2001). To perform this experiment we also reduced concentrations of Triton X-100 (25 μM) and SDS (10 μM) to avoid complete inhibition of GABA currents in the context of strong receptor activation. As shown in Figure 4A and B, depolarization did not significantly affect the inhibition by Triton X-100. In contrast, SDS inhibition exhibited weak but significant voltage dependence (Figure 4C and D). The voltage dependence was evident for both peak and late-phase GABA responses (Figure 4C and D). These results suggest that Triton X-100 behaves similarly to PS and to picrotoxin, which show no detectable voltage dependence (Akk *et al.*, 2001; Eisenman *et al.*, 2003), whereas SDS inhibition is more similar to pregnane steroids with a carboxylate or sulphate substituent at carbon 3 (Mennerick *et al.*, 2001).

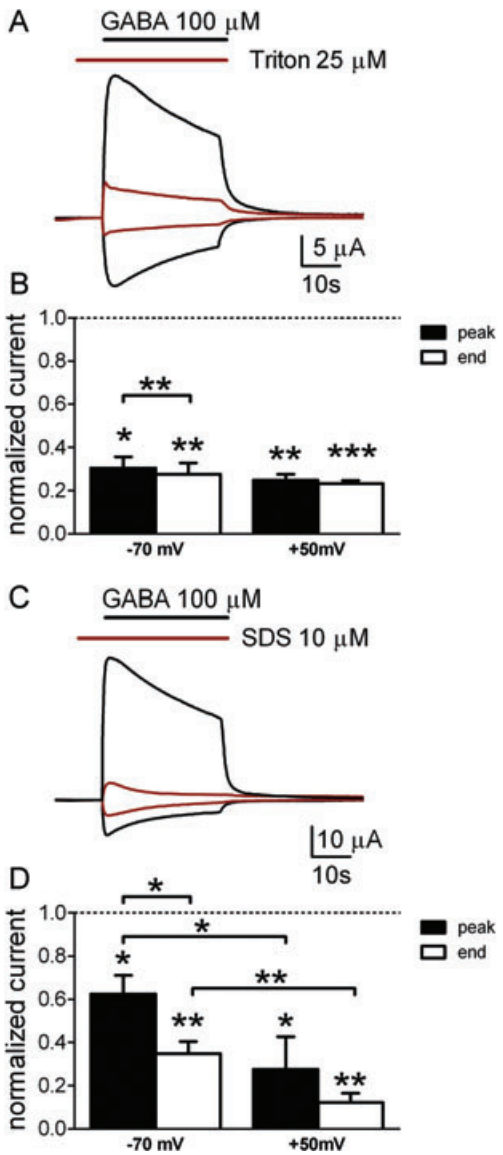

Figure 4 Inhibition is modestly voltage-dependent. (A and C) Representative oocyte currents to 100 μM GABA (black trace) and to co-application of Triton or sodium dodecyl sulphate (SDS) (red traces, as indicated). (B and D) Summary of normalized current (peak and end of drug application) in oocytes treated as in (A and C). Dotted horizontal line represents averaged GABA responses before and after drug application. Values are means ± SE (Triton, $n = 5$; SDS, $n = 6$), $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ (paired *t*-test), significantly different from responses to GABA alone (dotted line).

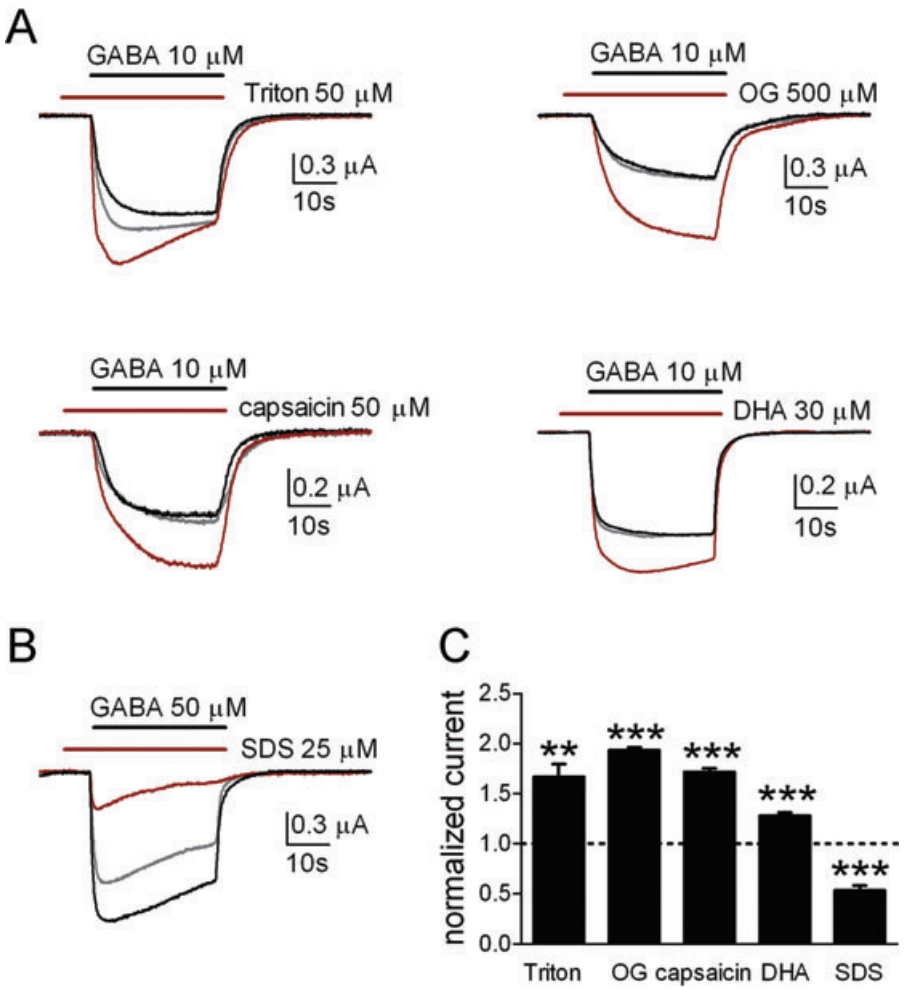

Figure 5 α1Q241L-mutated receptors show similar potentiation (and inhibition) as wild-type (WT) receptors. (A) Representative traces of GABA potentiation by amphiphiles (as indicated). (B) Representative traces of inhibited GABA current by sodium dodecyl sulphate (SDS) 25 μM. (C) Summary of peak current in oocytes treated as in (A and B). Dotted horizontal line represents averaged GABA responses before and after drug application. Values are means ± SE [Triton, $n = 6$; octyl-β-glucoside (OG), $n = 7$, capsaicin, $n = 6$; docosahexaenoic acid (DHA), $n = 7$; SDS, $n = 7$], $^{**}P < 0.01$, $^{***}P < 0.0001$ (paired *t*-test), significantly different from responses to GABA alone (dotted line).

Effects of amphiphiles in mutated $GABA_A$ receptors

Mutations in the α1 subunit affect positive and negative modulation by neurosteroids. For instance, residue Q241 in the M1 domain and residues N407 and Y410 in M4 domain of the α1 subunit have been proposed to be important sites for the interaction of potentiating 3α-hydroxysteroids with the receptor (Hosie *et al.*, 2006). By contrast, the M2 residue V256 likely is not directly involved in binding sulphated steroids. Rather, it helps transduce steroid binding to altered receptor function (Akk *et al.*, 2001). Mutation of this residue leaves picrotoxin inhibition intact (Akk *et al.*, 2001). We investigated the modulation by amphiphiles in mutated receptors expressing rat α1Q241L, α1N407A/Y410F and α1V256S along with WT β2 and γ2L subunits in oocytes. Each of these mutations affects the GABA concentration–response relationship, in addition to their effects on neurosteroid modulation (Figure S2). We adjusted GABA concentration in the various experiments so that potentiation and inhibition would be accentuated appropriately, as with WT receptors (see *Methods*).

We used the same protocol described previously in oocytes injected with α1Q241L subunit, along with WT β2 and γ2L. As predicted, co-application of a low, sub-EC_{50} GABA concentration (10 μM) and 3α5αP in α1Q241L-mutated receptors nearly eliminated the potentiation compared with that observed in WT receptors (Figure S3A and B). With the Q241L mutation, we found that in the presence of a low GABA concentration, Triton X-100, OG, capsaicin and DHA still potentiated GABA current (Figure 5A and C). Comparing potentiation by Triton X-100 in WT and α1Q241L-mutated receptors, there was a statistically significant reduction in potentiation in receptors expressing α1Q241L (Table 1). Nevertheless, this difference was very small and could be explained by slight differences in the effective concentrations chosen for the WT and mutated receptors respectively. No other amphiphile exhibited a significant change from WT potentiation. To test whether the Q241L mutation affected inhibition by amphiphiles, we co-applied a high GABA concentration (50 μM) and SDS. No difference from WT inhibition was detected (Figure 5B and C; Table 1).

We also tested the double mutated α1N407A/Y410F subunits, which also reduce the potentiation by 3α-hydroxysteroids (Hosie *et al.*, 2006; Akk *et al.*, 2008). These residues are proposed to represent the opposite end of a docking site for positive neurosteroid modulators (Hosie *et al.*, 2006). As with the Q241L mutation, the degree of potentiation observed in the double mutant for Triton X-100, OG, capsaicin and DHA with low GABA concentration (3 μM) was comparable to the WT subunit (Table 1).

We next considered the mutation α1V256S, which removes inhibition by sulphated steroids (Akk *et al.*, 2001; Eisenman *et al.*, 2003) (Figure S3C and D). Non-competitive antagonism by picrotoxin, which like PS shows voltage-independent, activation-dependent antagonism, was unaffected by the mutation (Figure S3E), as previously described (Akk *et al.*, 2001). Co-application of a high GABA concentration (5 μM) and Triton X-100 in oocytes expressing the α1V256S subunit revealed that the antagonism exhibited in WT receptors was nearly completely removed (Figure 6A and B vs. Figure 2A and B). In place of inhibition, we detected a small but statistically significant potentiation (Figure 6A and B) in mutated receptors (Table 1). There was also a weak residual enhancement of desensitization with Triton X-100 co-application (Figure 6B), which could suggest that inhibition was not completely abolished by the mutation. Alternatively, the weak increase in desensitization could be a direct consequence of potentiated receptor function (Figure 6A and B).

The other amphiphiles also exhibited strongly reduced negative modulation (Figure 6; Table 1). With SDS, the enhanced desensitization at high GABA concentration was markedly diminished compared with WT responses (Figure 6A and B vs. Figure 2A and B; Table 1). Potentiation observed for OG in WT receptors was still intact in α1V256S-mutated receptors, but as in Q241L-mutated receptors, the extent of potentiated GABA current was less than WT (Table 1). For capsaicin and DHA, no change in effects on peak current was detected compared with WT receptors, but the increased desensitization during drug application to WT receptors was removed (Figure 6B). In summary, our results showed that a mutation affecting negative modulation by neurosteroids also strongly reduced or eliminated negative modulation by structurally diverse amphiphiles.

To ensure that amphiphiles were still capable of interacting with α1V256S-containing receptors, we tested whether amphiphile potentiation was intact in the context of the V256S mutation. At low GABA concentration (0.05 μM), amphiphiles that potentiated GABA current in WT receptors (Triton X-100, OG, capsaicin and DHA), also strongly potentiated responses in oocytes expressing the α1V256S subunit (Figure 6C and D). Potentiation by Triton X-100, OG and DHA was actually stronger in presence of α1V256S subunit than with WT (Table 1). Because the differences between WT and V256S were strongest for Triton X-100 and SDS, we examined other concentrations of both modulators at the established low GABA concentrations. For Triton X-100, in neither receptor type did we observe significant potentiation at concentrations of 3–20 μM. Also, potentiation was similar in WT and V256S receptors at 30 μM (2.5 ± 0.3 for WT and 2.3 ± 0.1, normalized current values). Increasing Triton X-100 concentration to 50 and 100 μM, the difference in potentiation between WT and the V256S mutation was stronger (at 50 μM, WT 4.1 ± 0.4 vs. V256S 5.2 ± 0.5 normalized current values; at 100 μM WT 4.5 ± 0.3 vs. V256S 7.9 ± 0.6 normalized current values). This difference is likely to be explained by inhibition of WT currents apparent at high Triton concentrations, which is relieved in the V256S mutation. SDS application on α1V256S-mutated receptors in presence of 0.05 μM GABA showed a loss of inhibition (Figure 6C and D; Table 1), compared with WT receptors. There was also a statistically significant but small apparent SDS potentiation in some cells (Figure 6C and D). At 100 μM in V256S receptors, SDS still did not inhibit responses (1.2 ± 0.1, normalized current values). In the same experimental conditions, 1–10 μM PS showed a similar lack of inhibition in α1V256S-mutated receptors; no potentiation of responses was evident (Figure S4D and data not shown). Thus, amphiphile potentiation was intact in the α1V256S-mutated receptor; inhibition was eliminated at the highest concentrations tested.

Discussion and conclusions

Amphiphilic compounds tested in this study clearly have multiple actions on $GABA_A$ receptors. The structurally diverse compounds share a common action of decreasing bilayer stiffness and increasing elasticity (Lundbaek *et al.*, 1996); it is possible this non-specific action may underlie some of the common actions of the compounds. Recently, these structurally unrelated amphiphiles were all suggested to be $GABA_A$ receptor potentiators (Sogaard *et al.*, 2006; Yang and Sonner, 2008). Our work suggests considerably more complexity in amphiphile actions, with several compounds capable of both positive and negative modulation of GABA receptor function, and these opposing effects were clearly dissociable with GABA concentration and with mutated receptor subunits. Neurosteroid actions exhibit a number of superficial similarities with amphiphile modulation. Although neurosteroids likely have specific binding sites, the sites for these steroids are incompletely characterized. This is particularly true for sulphated GABA receptor-antagonist steroids. Furthermore, neurosteroids partition into the bilayer and could have non-specific effects akin to surfactants. Therefore, it is conceivable that some positive or negative modulatory effects of neurosteroids could be mediated through non-specific effects on membrane stiffness. We explored whether GABA current modulation by amphiphiles and by neurosteroids is mechanistically similar. We found that negative modulation but not positive modulation by amphiphiles shares a number of features with modulation by neurosteroids.

Potentiation

It seems likely that amphiphile hydrocarbon chain length is an important variable for potentiation, although the polar head group may also have an influence. It is interesting that OG has the shortest carbon tail (8 carbon atoms) compared with the other compounds tested, and co-application with GABA caused most robust potentiation of all the amphiphiles. Potentiation was evident at $<EC_3$ GABA concentration, as well as a GABA concentration $>EC_{50}$. This may suggest that the

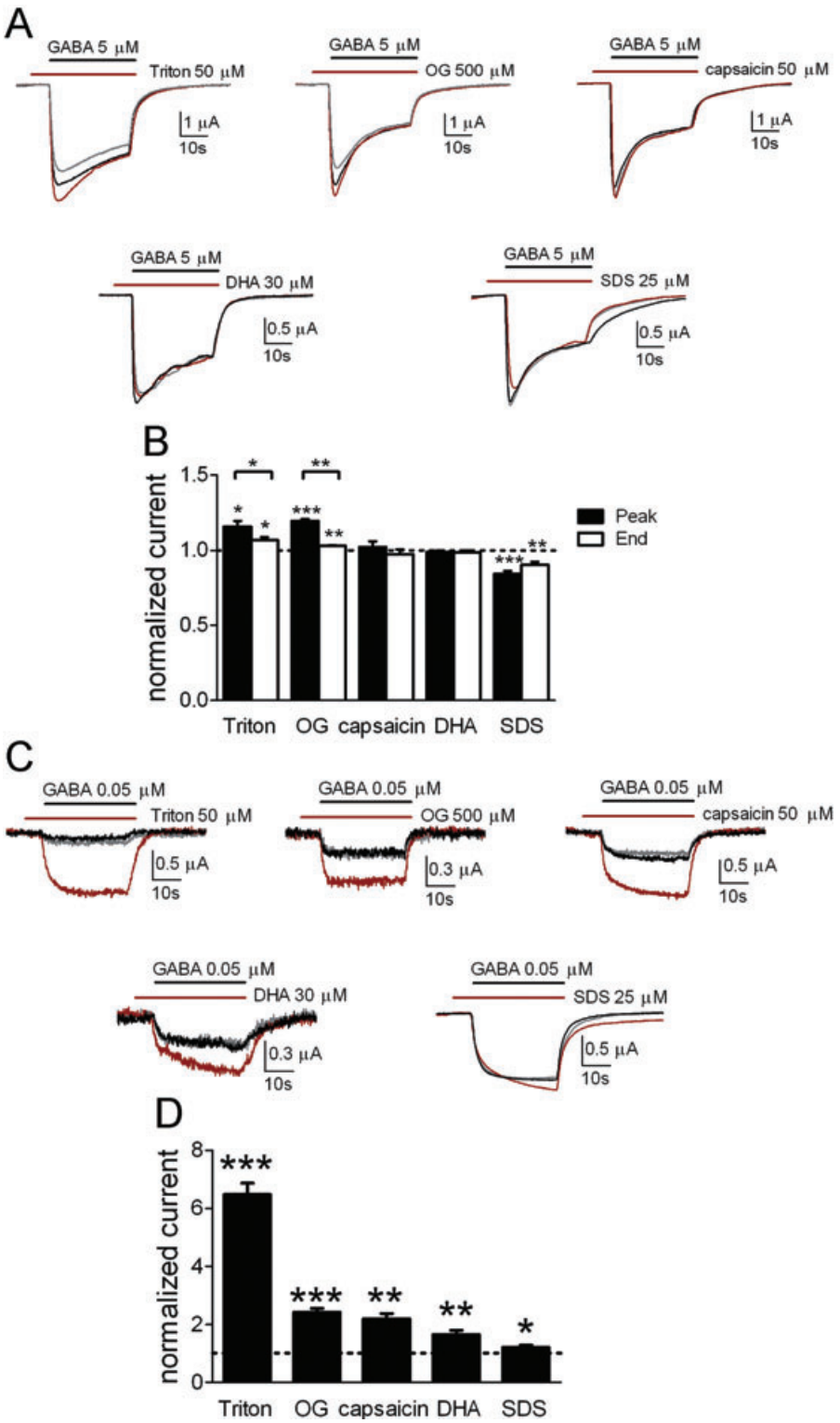

Figure 6 α1V256S mutation reduces amphiphile inhibition but not potentiation compared with wild-type (WT). (A) Representative responses of oocytes to 5 μM GABA (black traces) and to co-application of amphiphiles (red traces, as indicated). (B) Summary of normalized current (peak and steady state) in oocytes treated as in panel (A). Dotted horizontal line represents averaged GABA responses before and after drug application. Values are means ± SE [Triton, $n = 5$; sodium dodecyl sulphate (SDS), $n = 6$; octyl-β-glucoside (OG), $n = 5$, capsaicin, $n = 5$; docosahexaenoic acid (DHA), $n = 8$], $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ (paired *t*-test), significantly different from responses to GABA alone (dotted line). (C) Representative traces of GABA potentiation by amphiphiles (as indicated) in presence of 0.05 μM GABA. (D) Summary of peak current in oocytes treated as in (A). Dotted horizontal line represents averaged GABA responses before and after drug application. Values are means ± SE (Triton, $n = 6$; OG, $n = 6$, capsaicin, $n = 7$; DHA, $n = 7$; SDS $n = 7$), $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.0001$ (paired *t*-test), significantly different from responses to GABA alone (dotted line).

mechanism of potentiation for OG is different than other compounds. For other amphiphiles, the length of the hydrophobic tail varies between 11 and 31 atoms, and they show considerably weaker potentiation and variable inhibition.

There is precedent for an influence of the carbon tail length on the degree and the direction of modulation. For nicotinic acetylcholine receptors, an increase in carbon tail of n-alcohols changes enhancement to inhibition of current (Zuo *et al.*, 2001). These receptors have distinct binding sites of action for short- or long-chain alcohols (Wood *et al.*, 1991). In $5\text{-}HT_3$ receptors n-alcohols with a shorter chain (4–6 carbons) exhibit potentiation only at low concentrations but a negative modulation is observed at higher concentrations or with longer chains (up to 15 carbons) (Jenkins *et al.*, 1996). For $GABA_A$ receptors, increasing the carbon tail of n-alcohols results in increased current potentiation, up to a limit of 10–12 carbons, suggesting a receptor binding site than can accommodate only up to a certain chain length (Dildymayfield *et al.*, 1996). The structure of the amphiphiles tested here is more complex than n-alcohols, so other structural considerations may be more important in the case of the present amphiphiles. Channels known to be modulated by amphiphiles also include TREK-1 background potassium channels. These channels are positively modulated by amphiphiles (Honore, 2007). Increasing unsaturated chain length increases positive modulation, although amphiphile charge may also contribute (Patel *et al.*, 1998).

Identification of residues within the M1 and M4 domains of α1 subunit important for steroid potentiation (Q241 and N407/Y410) allowed us to test whether positive modulation by amphiphiles shares mechanisms with 3α-hydroxysteroids. Amphiphile potentiation was not substantially affected by mutation of either the M1 or M4 residues that strongly reduce neurosteroid potentiation. Inhibition (antagonism) also remained intact. These results support previous conclusions that positive and negative modulation by steroids involve different portions of $GABA_A$ receptors (Park-chung *et al.*, 1999; Akk *et al.*, 2001) and suggest that amphiphile modulation is dissimilar from neurosteroid potentiation. Our data do not allow us to distinguish whether a specific binding site is involved in potentiation by any of the amphiphiles. However, if receptor sites underlie amphiphile potentiation of GABA receptor function, they are apparently distinct from neurosteroid potentiation sites. This contrasts with another novel class of lipophilic potentiators, cembranoids, for which potentiation was removed by α1Q241L mutation (Li *et al.*, 2008).

Antagonism

In our results, SDS was the only compound that potently inhibited GABA current under all conditions and GABA concentrations. We suspect that the negative charge on SDS is partly responsible for strong antagonism by SDS, similar to sulphated neurosteroids. However, because DHA is also negatively charged, the shorter hydrocarbon chain of SDS may also be important for strong inhibition. We observed inhibition with as low as 5 μM SDS (data not shown). The less polar amphiphiles were weaker inhibitors. Similarly, we have previously found that uncharged, 3β-hydroxysteroids antagonize $GABA_A$ receptors by a mechanism indistinguishable from sulphated steroid block, albeit with lower potency than negatively charged, sulphated steroids (Wang *et al.*, 2002). A previous report suggested that SDS potentiates $GABA_A$ receptor function (Yang and Sonner, 2008). The reasons for the discrepancy between this work and ours are unclear.

Our results also apparently conflict with a recent report suggesting that Triton X-100 purely inhibits GABA responses (Sogaard *et al.*, 2009). Like us, this group observed that inhibition was characterized by an apparent enhancement of desensitization, but no overt potentiation was reported. This is likely because the previous report focused on the effects of 10 μM Triton X-100 and responses to elevated concentrations of GABA. Furthermore, our results suggest that potentiation by Triton X-100 is very transient when GABA and Triton are co-applied (Figure 3). Therefore, the potentiation phase of Triton X-100's effects could be easily missed.

With high GABA concentration, amphiphiles show inhibition in peak current, followed by enhanced desensitization, especially apparent with SDS and Triton X-100. Although open channel block (Adams, 1976; Neher and Steinbach, 1978; Huettner and Bean, 1988) is one explanation for the GABA concentration-dependent antagonism, other mechanisms could also account for the activation dependence of amphiphile antagonism (Eisenman *et al.*, 2003). Regardless of the exact mechanism, the behaviour is reminiscent of sulphated steroid antagonism of $GABA_A$ receptors (Eisenman *et al.*, 2003). Moreover, surfactant antagonism shows variable, weak voltage dependence, which is also similar to sulphated and carboxylated steroids (Mennerick *et al.*, 2001). As a strong test of the similarity of mechanism between surfactants and sulphated neurosteroids, we examined a mutated receptor that is insensitive to sulphated steroid modulation but sensitive to another activation-dependent antagonist, picrotoxin. The mutation dramatically reduced amphiphile antagonism suggesting shared mechanisms between sulphated steroids and amphiphiles. Because the mutated residue is deep on the cytoplasmic side of M2, charged drugs like sulphated steroids probably do not reach this residue. Therefore, this residue is unlikely to mediate direct binding and is more likely to transduce a conformational change required for inhibition.

These results suggest that non-specific effects of amphiphiles and sulphated steroids may participate in antagonism of $GABA_A$ receptors, and are consistent with other evidence arguing for a lack of specificity, including relatively poor steroid enantioselectivity (Nilsson *et al.*, 1998). However, this interpretation is inconsistent with data from $GABA_A$ ρ1 receptors and from invertebrate GABA receptors, which suggest sulphated steroid enantioselectivity (Li *et al.*, 2006; Twede *et al.*, 2007). Therefore, specific binding sites for sulphated steroids may play a bigger role at some receptors than at others, and our results do not completely exclude the possibility of a site on $GABA_A$ receptors that mediates antagonism.

Taken together, these observations suggest that amphiphiles antagonize GABA receptors with effects similar to sulphated steroids. The similar mechanisms shared among compounds of varied structure suggest that negative steroid modulators might act by temporary modifications of the plasma membrane, such as changes in elasticity, that affect channel conformation.

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Statement of conflicts of interest

None.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Structures of tested amphiphiles and neurosteroids. (A) Triton X-100. (B) Octyl-β-D-glucopyranoside (OG). (C) Capsaicin. (D) *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA). (E) Sodium dodecyl sulphate (SDS). (F) (3α,5α)-3-hydroxypregnan-20-one (3α5αP). (G) Pregnenolone sulphate (PS).

Figure S2 Mutations modify $GABA_A$ receptor sensitivity. (A) GABA concentration–response curves in wild-type (WT) (filled circles, $n = 5$) and mutated (V256S, open squares, $n = 5$; Q241L, filled squares, $n = 5$; N407A/Y410F, open circles, $n = 4$) $GABA_A$ receptor α subunits. Responses were expressed relative to the response of the highest GABA concentration applied (WT, 100 μM; V256S 10 μM; N407A/Y410F and Q241L, 300 μM) to facilitate comparison of shapes and EC_{50} values. The solid lines represent fits of the Hill equation to the averaged data points shown in the figure. Parameters from the summary fits of WT, V256S-, N407A/Y410F- and Q241L-mutated α subunits respectively were: EC_{50} 12.8 μM, 1.4 μM, 20.5 μM and 37.6 μM; Hill coefficient: 1.3, 2.6, 1.6, 2.1. (B) Representative responses from oocytes expressing WT and V256S-mutated receptors challenged with increasing GABA concentrations (WT, 0.3–100 μM; V256S, 0.03–10 μM).

Figure S3 Modulation by neuroactive steroids is altered in mutated $GABA_A$ receptors. (A and B) Representative traces of (3α,5α)-3-hydroxypregnan-20-one (3α5αP) potentiation on wild-type (WT) (A) and α1Q241L-mutated (B) receptors. (C and E) Representative traces of pregnenolone sulphate (PS) inhibition on WT (C) and α1V256S-mutated (D) receptors. As shown, PS inhibition is removed in presence of α1V256S subunit (D). The same mutation does not affect inhibition by 20 μM picrotoxin (E).