Ethanol modulates the interaction of the endogenous neurosteroid allopregnanolone with the α1β2γ2L GABA<sub>A</sub> receptor

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Running title - ethanol and steroid interactions on the GABA<sub>A</sub> receptor

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Number of text pages: 36
Number of tables: 4
Number of figures: 7
Number of references: 37
Number of words in Abstract: 235
Number of words in Introduction: 762
Number of words in Discussion: 1628

Abbreviations:

3α5αP, allopregnanolone; 17-PA, (3α,5α)-17-phenylandrost-16-en-3-ol; ACN, (3α,5α,17β)-3-hydroxyandrostane-17-carbonitrile;
ABSTRACT

We have examined the α1β2γ2L GABA<sub>A</sub> receptor modulation by the endogenous steroids allopregnanolone (3α5αP), pregnenolone sulfate, and β-estradiol in the absence and presence of ethanol. Coapplication of 0.1-1 % (17-170 mM) ethanol influenced receptor modulation by 3α5αP but not by pregnenolone sulfate or β-estradiol. One of the three kinetic effects evident in channel potentiation by 3α5αP, prolongation of the longest-lived open time component (OT3), was affected by ethanol with the midpoint of its dose-response curve moved to lower steroid concentrations by two orders of magnitude without significantly affecting the maximal effect. Manipulations designed to affect the ability of 3α5αP to prolong OT3 also affected OT3 prolongation in the presence of ethanol. A mutation to the γ2 subunit, which reduces the ability of 3α5αP to prolong OT3, also reduces the interaction between ethanol and 3α5αP. And the presence of the competitive steroid antagonist, (3α,5α)-17-phenylandrost-16-en-3-ol (17-PA), diminishes the positive interaction between ethanol and 3α5αP on the GABA<sub>A</sub> receptor. Together, the findings suggest that steroid interactions with the classic steroid binding site underlie the effect seen in the presence of ethanol, and that ethanol acts by increasing the affinity of 3α5αP for the site. Tadpole behavioral assays showed that the presence of 3α5αP at a concentration ineffective at causing changes in tadpole behavior shifted the ethanol dose-response curve for loss of righting reflex to lower concentrations, and that this effect was neutralized by coapplication of 17-PA with 3α5αP.
INTRODUCTION

From a pharmacologist’s point of view, the GABA$_A$ receptor presents a fascinating case because the receptor can be modulated by a wide collection of structurally unrelated drugs, most with their own individual binding sites. The GABA$_A$ receptor constitutes the dominant fast inhibitory force in the central nervous system. Unsurprisingly, compounds which potentiate receptor function have anesthetic or anticonvulsant properties, and conversely, inhibitors of the GABA$_A$ receptor can act as cognitive enhancers or elicit seizures. The clinical implications have, no doubt, fueled the interest in receptor function resulting in rapid progress in recent years in the identification of interaction sites and mechanisms of action for the modulators of the GABA$_A$ receptor.

Neuroactive steroids are among the best-described GABA$_A$ receptor modulators. The ability of steroids to modify GABA$_A$ receptor activity has been known since the 1980s (Harrison and Simmonds, 1984), and since then significant progress has been made in terms of describing the kinetic and structural mechanisms (e.g., Puia et al., 1990; Akk et al., 2004; Hosie et al., 2006) as well as understanding the physiological significance of steroid modulation (for reviews see, Baulieu, 1998; Belelli and Lambert, 2005). Exposure to most steroids has a major effect on gating or desensitization of the receptor-channel. Potentiating steroids (e.g., 3α5αP) enhance the channel open probability via changes in the gating properties (Twyman and Macdonald, 1992; Bianchi and Macdonald, 2003; Akk et al., 2004). The mean open time is increased and the mean closed time decreased as a result of steroid application. This boosts the membrane conductance to Cl$^-$, ultimately resulting in reduction of cellular excitability. In contrast, inhibitory steroids (e.g., pregnenolone sulfate) act by enhancing the rate of desensitization (Shen et al., 2000; Akk et al., 2001). This results in premature termination of the GABA$_A$ receptor response, thereby shifting the balance between excitation and inhibition in the direction of excitation.
The ability of ethanol to modulate GABA_A receptor function has been much less well-studied, in major part due to the difficulties involved in observing a robust effect. While ethanol may act indirectly on the GABA_A receptor, via changes in the levels of other receptor modulators (VanDoren et al., 2000), direct functional modulation of the GABA_A receptor by ethanol has been more difficult to observe, and has been proposed to depend on the subunit composition of the receptor (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003 but see Borghese et al., 2005), phosphorylation (Wafford and Whiting, 1992 but see Zhai et al., 1998) or the presence of cofactors (Akk and Steinbach, 2003). It has recently been shown that exposure to alcohol induces a conformational change in the GABA_A receptor structure, and that mutagenesis of specific residues in the M2 and M3 domains interferes with this process (Jung and Harris, 2006). This, quite possibly, provides the best proof so far of direct action of ethanol on the GABA_A receptor.

It is likely that, in most cases in vivo, the GABA_A receptor is simultaneously exposed to multiple potential modulators whose cumulative effect may not be simply determined based on the individual drug effects. Instead, allosteric interactions between sites for different modulators may form the basis for endogenous modulation. Thus, in order to gain insight into the modulation of the receptor under physiological conditions, it is necessary to study not only the mechanisms of action of individual compounds but to also explore the interplay between the actions of drugs.

We have previously shown that ethanol and a synthetic neuroactive steroid (3α,5α,17β)-3-hydroxyandrostane-17-carbonitrile (ACN) act synergistically to potentiate the function of the α1β2γ2L GABA_A receptor (Akk and Steinbach, 2003). The presence of ethanol shifted the dose-response curve of the steroid toward lower concentrations, and resulted in receptor potentiation at steroid concentrations previously incapable of modifying channel function. Only the effect on the mean open time by the steroid was
affected by ethanol, raising the possibility that ethanol interaction with the receptor influences just one of the several interaction sites postulated for neuroactive steroids. It should be noted that ethanol alone had no appreciable effect on single-channel currents.

Here, we describe an examination of the interactions between ethanol and endogenous neurosteroids with differing modes of action (3α5αP, pregnenolone sulfate and β-estradiol) on the α1β2γ2L GABA<sub>A</sub> receptor. Our results demonstrate that ethanol interactions with the receptor modulate only one component of potentiating actions of 3α5αP, most likely by enhancing the affinity of steroid for the site. Ethanol was ineffective at modifying channel modulation by pregnenolone sulfate or β-estradiol. The electrophysiological studies were complemented with tadpole behavioral assays which demonstrated that 3α5αP shifts the ethanol dose-response curve for loss of righting reflex to lower concentrations.

**MATERIALS AND METHODS**

*Molecular biology and cell culture*

The rat GABA<sub>A</sub> receptor subunit clones were provided by Drs. A. Tobin (α1, β2) and D. Weiss (γ2L), and subcloned into a CMV promoter-based vector, pcDNAIII (Invitrogen, San Diego, CA). The receptors were expressed transiently in HEK 293 cells using a calcium phosphate precipitation-based transfection technique (Akk, 2002). A total of 6 µg of cDNA per 35 mm culture dish was used in the ratio of 1:1:1 for α:β:γ. The cells were incubated with the precipitate for 16-20 hours, after which the medium in the dish was replaced. The electrophysiological experiments were carried out during the next two days after changing the medium.
Electrophysiology

The electrophysiological experiments were carried out using the cell-attached patch clamp and whole-cell voltage clamp techniques. The bath solution contained (in mM): 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 10 glucose and 10 HEPES; pH 7.4. In single-channel recordings, the pipet solution contained (in mM): 120 NaCl, 5 KCl, 10 MgCl₂, 0.1 CaCl₂, 20 tetraethylammonium, 5 4-aminopyridine, 10 glucose, 10 HEPES; pH 7.4. In whole-cell recordings, the pipet solution contained (in mM): 140 CsCl, 4 NaCl, 4 MgCl₂, 0.5 CaCl₂, 5 EGTA, 10 HEPES, pH 7.4.

The receptor agonist (GABA), steroidal modulators (3α5αP, pregnenolone sulfate or β-estradiol) and ethanol were added to the pipet solution in single-channel recordings, or applied through the bath using an SF-77B fast perfusion stepper system (Warner Instruments, Hamden, CT) in whole-cell experiments. The steroids were initially dissolved in DMSO at 10 mM concentration, and diluted immediately before the experiment.

In single-channel recordings, the pipet potential was normally held at +60 to +80 mV, which translates to an approximately -120 to -100 mV potential difference across the patch membrane. In whole-cell recordings, the cells were clamped at -60 to -20 mV. The membrane current was recorded using an Axopatch 200B amplifier, low-pass filtered at 10 kHz, and acquired with a Digidata 1320 series interface at 50 kHz using pClamp software (Molecular Devices, Union City, CA). The whole-cell current traces were low-pass filtered at 2 kHz and acquired at 10 kHz. The data were stored on a PC hard drive for further analysis.

Data analysis

The strategy for the analysis of single-channel and whole-cell currents has been described in detail previously (Akk et al., 2001; Li et al., 2006). The key features of the
analysis are the following. The analysis of single-channel currents was limited to clusters, i.e., episodes of intense activity originating from the activation of a single ion channel, or fragments of clusters containing no overlapping currents. The currents were low-pass filtered at 2-3 kHz, and the data were idealized using the segmented-k-means algorithm (Qin et al., 1996). The analysis consisted of the determination of open and closed times which were estimated from the idealized currents using a maximum likelihood method which incorporates a correction for missed events (QuB Suite; www.qub.buffalo.edu).

The analysis of whole-cell currents was directed at determining the peak current amplitude and the desensitization and deactivation time constants using the pClamp 9.0 software package.

**Tadpole behavioral assays**

Groups of 10 early prelimb-bud stage *Xenopus laevis* tadpoles (Nasco, Fort Atkinson, WI) were placed in 100 ml of oxygenated Ringer’s solution containing steroids (3α5αP and/or 17-PA). After equilibration for 3 hrs, the tadpoles were evaluated using the loss of righting reflex (LRR) behavioral endpoint which was defined as the failure of the tadpole to right itself within 5 s after being flipped by a glass rod. Appropriate amounts of ethanol were then added to the beakers, tadpoles allowed to equilibrate for 15 minutes, and LRR measured again. In experiments where ethanol was used without steroids, the first 3 hr incubation was skipped. In each case, the tadpole Ringer's solution contained 0.1 % DMSO originating from the steroid stock solutions or, when ethanol was used in the absence of steroids, added separately for consistency. Control beakers containing 0.1 % DMSO produced no LRR in tadpoles.

**Drugs**

Most drugs were purchased from Sigma Chemical Co. (St. Louis, MO). Ethanol
was purchased from Aaper Alcohol and Chemical Co (Shelbyville, KY). 17-PA was a gift from Dr. D. F. Covey.

RESULTS

**Kinetic mechanisms of GABA<sub>A</sub> receptor modulation by neuroactive steroids**

At GABA concentrations at or above 20 µM, the channel activity from the α1β2γ2L receptor takes place in clusters, i.e., episodes of intense activity. There is a great deal of complexity in the intracluster kinetics, and many of the fine features of channel activation are still poorly understood. The consensus at the present time (e.g., Fisher and Macdonald, 1997; Steinbach and Akk, 2001; Lema and Auerbach, 2006) is that the channel openings from αβγ receptors contain three classes of openings, distinguishable by their mean durations and relative frequencies (OT1 – brief, OT2 – intermediate and OT3 – long), but presumably all originating from fully-liganded states (Figure 1A top trace). The closed time distributions contain three or four components, depending on the agonist concentration, reflecting dwells in the activation pathway, i.e., channel closing, agonist dissociation, re-binding, re-opening, and in states distal to the open states, such as short-lived blocked or desensitized states. The activation scheme for the receptor is still not fully established, although an agreement on many key features of the activation properties seems to have been reached (Lema and Auerbach, 2006).

We have previously identified three kinetically distinct effects in the modulation of the α1β2γ2L receptor by potentiating neuroactive steroids (Akk et al., 2004; Akk et al., 2005). The presence of ACN or 3α5αP, coapplied with 50 µM GABA, results in a dose-dependent increase in the mean duration and relative frequency of the longest-lived open time component (OT3) within clusters. The mean duration of OT3 increases from around 6 ms to 15-20 ms, and its prevalence increases from around 10 % to 40 %. As a result,
the mean open duration of the channel is increased by 2-3-fold in the presence of saturating concentrations of ACN or 3α5αP. In addition to that, exposure to potentiating steroids results in a decrease in the relative frequency of the activation-related closed time component (CTβ) as a result of reduction of the channel closing rate. Since CTβ is the longest-lived intracluster closed time component at 50 µM GABA, a decrease in its contribution produces a decrease in the mean closed time duration. Together, the increase in the mean open time and the decrease in the mean closed time contribute to the overall potentiating effect of a steroid.

Inhibitory steroids such as pregnenolone sulfate do not influence the intracluster open or closed time distributions. Instead, the presence of pregnenolone sulfate increases the apparent rate of desensitization resulting in a reduction in the mean cluster duration (Akk et al., 2001).

**Coapplication of ethanol with 3α5αP results in potentiation of single-channel currents elicited by 50 µM GABA**

In order to evaluate the effect of ethanol on GABA_A receptor function, we coapplied 0.01-1 % ethanol (1.7-170 mM) along with 50 µM GABA in the presence of 10 nM 3α5αP (Figure 1). At this concentration, 3α5αP is ineffective at modulating GABA-activated currents (Figures 1A, 2; Akk et al., 2005), and our previous study has shown that 0.1-2 % (17-340 mM) ethanol applied in the absence of steroid potentiators also does not modulate receptor activation by GABA (Akk and Steinbach, 2003). However, when 10 nM 3α5αP was coapplied with ethanol, the effect was one of potentiation involving a single parameter of steroid-mediated potentiation. Ethanol prolonged the mean duration of OT3 in a dose-dependent manner with an EC_{50} at 0.015 % (2.6 mM) in the presence of 3α5αP but was essentially ineffective at producing changes in the
prevalence of OT3 or CTβ (Figure 1B).

We next tested concentrations of 3α5αP capable of producing an increase in the duration of OT3. Figure 2 shows the 3α5αP dose-response curves in the absence (control) and presence of 0.1 % (17 mM) ethanol. Compared to the control conditions, the presence of ethanol leads to a major leftward shift in the steroid dose-response curve for prolongation of OT3 without significant changes in the maximal level. The EC$_{50}$ for the curve was at 66 nM in the absence of ethanol and at 0.52 nM in the presence of ethanol. In contrast, the presence of ethanol produced only minor changes in the steroid dose-response curve for the fraction of CTβ (Figure 2C) while rather prominent scatter among the data points prevented detailed analysis of the effect of ethanol on the fraction of OT3 (Figure 2B).

A steroid antagonist, 17-PA, blocks the increase in the duration of OT3

(3α,5α)-17-phenylandrost-16-en-3-ol (17-PA) is a steroid analog which selectively antagonizes potentiation by 5α-reduced neuroactive steroids and whose mechanism of action involves both competitive and noncompetitive components (Mennerick et al., 2004).

Our single-channel studies show that the major effect of 17-PA, when coapplied with 50 μM GABA and 1 μM 3α5αP, is to reduce the prolongation of OT3 duration without affecting the increase in the fraction of OT3 or the reduction in the fraction of CTβ. The mean duration for OT3 was reduced from 14.1±2.1 ms (n=3 patches) to 9.4±3.7 ms (n=5 patches) when 10 μM 17-PA was coapplied with GABA and 3α5αP. In contrast, the fractions of OT3 (38±4 % vs. 33±11 %) and CTβ (5±1 % vs. 8±2 %) remained relatively constant in the absence and presence of 17-PA.

In order to test the involvement of classic steroid binding sites in ethanol-induced
potentiation we have examined the ability of 17-PA to block the prolongation of OT3 observed in the presence of ethanol. This experiment is based on the idea that 17-PA would prevent an increase in OT3 duration in the presence of ethanol + steroid if the increase results from steroid interactions with the classic steroid interaction site but would be ineffective if the presence of ethanol reveals a novel interaction site for $3\alpha5\alpha P$.

The results of the analysis of single-channel recordings are summarized in Table 1, and sample currents are shown in Figure 3. The results demonstrate that 10 µM 17-PA effectively blocks the increase in the OT3 duration observed in the presence of steroid+ethanol. The mean duration of OT3 was 18.0±4.5 ms (n=3 patches) at 10 nM $3\alpha5\alpha P + 0.1 \%$ ethanol but only 9.7±3.5 (n=7 patches) when 17-PA was coapplied with $3\alpha5\alpha P$ and ethanol. Thus, 17-PA similarly affects the prolongation of OT3 caused by low concentrations of $3\alpha5\alpha P$ in the presence of ethanol, and by high concentrations of $3\alpha5\alpha P$. Our interpretation of the finding is that the prolongation of OT3 is accomplished in both cases through steroid interactions with the same site, and that the presence of ethanol allosterically increases the affinity of the site to $3\alpha5\alpha P$ so that effects of the same magnitude are produced at 1 µM $3\alpha5\alpha P$, and 10 nM $3\alpha5\alpha P + ethanol$.

**A substitution in the C'-terminus of the γ2 subunit affects the ability of ethanol to prolong OT3 duration**

We have shown previously that substitution of the carboxy terminal portion of the γ2 subunit with homologous sequence from the ρ subunit selectively blocks the increase in the OT3 duration in the presence of ACN (Akk et al., 2004). One of the possible interpretations of the findings is that interactions of ACN (and, possibly, those of other potentiating steroids) with the C-terminus result in the prolongation of OT3, i.e., the C-terminus of the γ2 subunit forms a part of the steroid binding site.
If our hypothesis that steroid interactions with the classic interaction site underlie the effect seen in the presence of ethanol is correct, then the sequence substitution should also affect the ethanol-dependent prolongation of OT3. In contrast, if steroid interactions with a novel, hitherto undescribed binding site underlie the effect seen during coapplication with ethanol, then such a sequence swap would be ineffective, and ethanol would enhance the OT3 duration to the same level as seen in the wild-type receptor.

As the first step, we examined whether the C-terminal substitution affects channel potentiation by 3α5αP. Sample currents are shown in Figure 4, and the results are summarized in Table 2. In the presence of 50 µM GABA, the mean OT3 duration was 6.9 ms, which was increased to 7.9 ms in the presence of GABA + 0.1 µM 3α5αP, and to 10.6 ms in the presence of GABA + 1 µM 3α5αP. This is to be compared to the wild-type receptor for which the mean duration of OT3 was increased from roughly the same baseline value (6-7 ms) to 14.1 ms (see above) in the presence of 1 µM 3α5αP. Thus, the amino acid sequence substitution leads to reduction in the maximal effect of 3α5αP on OT3 duration.

In the second set of experiments, we tested whether 0.1 % (17 mM) ethanol, coapplied with GABA and 0.1 µM 3α5αP, was able to further increase the OT3 duration. The data show that the coapplication of ethanol prolonged the mean OT3 duration to 10.1 ms (Table 2). This value is similar to the OT3 duration for the mutant receptor observed in the presence of GABA + 1 µM 3α5αP, but significantly different ($p=0.038$) from the OT3 duration seen in the presence of steroid and ethanol in the wild-type receptor. Thus, the C'-terminal substitution affects prolongation of OT3 both in the presence of a high concentration of 3α5αP as well as in the presence of a low concentration of 3α5αP + ethanol. One interpretation of the results is that steroid interactions with the same site underlie prolongation of OT3 both in the absence and presence of ethanol. We would,
however, like to point out that a possible caveat to our interpretation of the data is that the mutation may also have affected receptor interactions with ethanol preventing a full-blown effect as seen in the wild-type receptor.

### Whole-cell recordings and tadpole behavioral assays in the presence of 3α5αP and ethanol

We next carried out whole-cell recordings in the presence of 5 μM GABA and 2 nM 3α5αP in the absence and presence of 0.1 % (17 mM) ethanol. This concentration of GABA corresponds to approximately EC\textsubscript{25} in the whole-cell dose-response curve (Li et al., 2006) and, thus, mimicks the 50 μM GABA used to activate the receptor in single-channel recordings. The concentrations for steroid and ethanol were selected from the single-channel data presented in Figures 1 and 2, and were expected to produce a maximal effect. To our surprise, the combination of 3α5αP and ethanol did not lead to potentiation of peak current or changes in other parameters of macroscopic currents (Figure 5). Compared to control currents obtained in the presence of GABA, the peak response was 100±15 % (n=6 cells) in the presence of ethanol alone, 101±8 % in the presence of 3α5αP alone, and 101±8 % when ethanol and 3α5αP were coapplied. We carried out further macroscopic recordings varying the steroid concentration (1, 2 or 5 nM) while increasing the ethanol concentration to 1 % (170 mM). At no steroid concentration did the presence of ethanol produce a significant increase in peak current (data not shown).

In order to help us to evaluate the physiological effects of the allosteric interactions of 3α5αP and ethanol, we examined the anesthetic effects of ethanol in the absence and presence of steroids using Xenopus tadpoles. The EC\textsubscript{50}s for the LRR are presented in Table 3. The results demonstrate that the addition of 30 nM 3α5αP along
with ethanol shifts the ethanol dose-response curve from 228 mM to 107 mM. The steroid alone at this concentration was ineffective at causing LRR. The effect of 3α5αP was neutralized by the addition of 10 µM 17-PA resulting in an EC50 of 244 mM for the ethanol dose-response curve. Thus, the LRR is affected in a manner consistent with the hypothesis that ethanol and 3α5αP act synergistically on the GABA_A receptor, and that steroid interactions with a characterized potentiating site underlie the effect seen in the presence of ethanol.

**Ethanol does not modulate receptor function in the presence of pregnenolone sulfate**

Pregnenolone sulfate is an inhibitory steroid at the GABA_A receptor whose mode of action is to increase the apparent rate of desensitization. This results in shorter mean cluster duration but has no effect on the intracluster open and closed time distributions. It has been proposed that this steroid does not interact with the sites mediating channel potentiation as receptors exposed simultaneously to pregnenolone sulfate and ACN exhibited properties characteristic of both inhibition (short cluster) and potentiation (prolonged openings) (Akk et al., 2001). Accordingly, we would not expect any interaction between ethanol and pregnenolone sulfate.

The control currents were recorded in the presence of 50 µM GABA and 25 µM pregnenolone sulfate. In agreement with previous studies, exposure to pregnenolone sulfate resulted in reduction in cluster length without affecting the intracluster open or closed time distributions. Sample clusters are shown in Figure 6A and the summary of open and closed time analysis is given in Table 4. Coapplication of 1 % (170 mM) ethanol along with GABA and pregnenolone sulfate had only minor effects on the intracluster properties (Table 4). We conclude that ethanol does not elicit an effect characteristic of
3α5αP when pregnenolone sulfate is used as the steroidal modulator.

We examined whether ethanol affects receptor desensitization in the presence of pregnenolone sulfate using whole-cell recordings. In these experiments, we compared the properties of whole-cell currents elicited by 50 μM GABA in the presence of 25 μM pregnenolone sulfate in the absence and presence of 1 % (170 mM) ethanol. A sample recording is shown in Figure 6B. Averaged data from 6 cells show that the desensitization time constant was 5.5±1.5 s in the presence of 50 μM GABA alone, and 0.26±0.04 s in the presence of GABA + 25 μM pregnenolone sulfate. Coapplication of 1 % ethanol with GABA and steroid did not affect the desensitization decay which remained at 0.26±0.03 s (n=6 cells). Similarly, there were only minuscule changes in the peak current response. Exposure to pregnenolone sulfate alone gave 94±11 % of the control (GABA alone) current while the application of steroid + ethanol gave 103±5 % of control current.

**Ethanol does not modulate receptor function in the presence of β-estradiol**

β-Estradiol is a female sex hormone whose main role lies in gene transcription regulation. Its effects on the GABA<sub>A</sub> receptor are poorly characterized but the steroid is believed to be largely ineffective at modulating receptor function (Zhang and Jackson, 1994).

Our goal was to examine whether β-estradiol and ethanol positively interact to modulate the GABA<sub>A</sub> receptor function. The lack of a potentiating effect of β-estradiol on the GABA<sub>A</sub> receptor currents does not necessarily result from its inability to interact with the steroid binding site. Other factors, such as low efficacy or competing mechanisms may contribute and result in the overall negligible influence of β-estradiol. If so, the presence of ethanol may sensitize the receptor to the actions of β-estradiol and reveal an interaction between the steroid and the receptor.
We initially examined receptor activation when β-estradiol was coapplied with GABA. The single-channel currents showed changes in both open and closed time distributions (Figure 7A, Table 4). The relative contribution of OT3 was enhanced in the presence of β-estradiol, although this difference did not reach statistical significance. In addition, the apparent duration of CTβ was increased, and in 3 of 4 patches, the closed time distributions were significantly better fitted to a sum of four exponentials. This resulted in minor changes in the two shorter duration components (CT1 and CT2) but the nominal CTβ was split to two components, one with a mean lifetime of 9.7±1.5 ms and another with a mean duration of 34.4±3.7 ms.

We have previously shown that at 50 µM GABA, the class of longest-lived closed times actually contains two unrelated components (Steinbach and Akk, 2001). The majority of dwells are associated with the activation pathway, i.e., channel closing and then occupying the monoliganded and unliganded closed states before reopening, but a small portion (2 % of total) belongs to dwells in the short-lived desensitized state (henceforth called CTSD) (Jones and Westbrook, 1995) with a duration independent of the concentration of agonist. Since it is essentially impossible to distinguish the two components due to their close durations, and because of domination by one of the components, we have considered it fair to attribute all closed dwells of this class to the activation-related channel closures and essentially ignore the contributions from the short-lived desensitized state. The present data, however, indicate that this approach is unsuitable for describing channel modulation by β-estradiol and that both components should be characterized individually.

The shorter-lived component (9.7 ms) formed 27 % and the longer-lived component (34.4 ms) formed 7 % of all intracluster closed events. The first of the two components is similar in its mean duration and relative weight to the CTβ component in
the absence of modulators (see Table 4), and, thus, likely to originate from dwells in unliganded and monoliganded closed states. The second, longer-lived component may represent sojourns in the CTSD state. If so, the presence of β-estradiol enhances the prevalence of this component (from ~2 % to ~7 % of total intracluster closed events), and doubles its duration.

We were curious to see whether the changes seen in single-channel currents translate into a measurable effect in whole-cell recordings. Data from six cells showed that 10 µM β-estradiol was ineffective at modifying the peak response (99±10 % of control response obtained in the presence of 10 µM GABA). Similarly, the presence of steroid did not affect strongly channel desensitization which had a time constant of 3.2±1.4 s under control conditions, and 3.3±1.6 s in the presence of GABA and β-estradiol. However, the application of steroid significantly affected the deactivation properties of the receptor (Figure 7B). The current deactivation time course was fitted to a single exponential decay with a mean value of 85±13 ms when responses were elicited with 10 µM GABA, this value increased to 169±40 ms (p=0.0006) after application of 10 µM GABA + 10 µM β-estradiol.

A previous study (Jones and Westbrook, 1995) has shown that following a pulse of GABA, the channels can enter a relatively long-lived desensitized state from which reopening can occur (see above), and proposed that sojourns in such nonconducting states slow the deactivation decay. Overall, our data would agree with these findings, and indicate that β-estradiol slows channel deactivation through an increase in the relative number of such events and, to a smaller extent, the lifetime of desensitized states.

Coapplication of 1 % (170 mM) ethanol with β-estradiol did not lead to further changes in receptor function. Sample current traces are shown in Figure 6 and the results from single-channel kinetic analysis are summarized in Table 4. In 4 of 5 patches, the
closed time distributions were best characterized by four components with parameters similar to those seen in the absence of ethanol. In whole-cell recordings, the application of ethanol did not modulate currents from receptors exposed to GABA and β-estradiol. The peak response was 106±20 % of control, the desensitization time constant was 3.9±2.0 s, and the deactivation time constant was 163±40 ms (n= 6 cells).

**DISCUSSION**

In this manuscript, we present the results from our studies on recombinant α1β2γ2L GABA<sub>λ</sub> receptor modulation by endogenous neurosteroids and ethanol. The steroids were selected to represent a spectrum of endogenous neurosteroid effects in the brain (potentiating, inhibitory and neutral). The overall goal of the work was to evaluate physiological significance of endogenous steroids in alcohol effects, and to correlate the distinct binding sites and mechanisms of the steroids with the ability to produce effect in the presence of ethanol. The crux of the paper is that the presence of low concentrations of ethanol (<0.1 %, 17 mM) sensitizes the receptor to 3α5αP, shifting the dose-response curve for a steroid effect by almost two orders of magnitude towards lower steroid concentrations. In addition, the data from experiments on Xenopus tadpoles show that the presence of low concentrations of 3α5αP enhances the anesthetic potency of ethanol suggesting that the ethanol site and a site for neurosteroids are mutual positive allosteric modulators. These findings are qualitatively different from the direct effects of ethanol seen at high concentrations in the absence of modulators (e.g., Mihic et al., 1997; Ueno et al., 1999).

Previous studies have demonstrated that GABA<sub>λ</sub> receptor potentiation by steroids is accomplished through modification of several kinetic parameters (e.g., Twyman and
We have proposed that steroid interactions with at least two sites on the receptor underlie three kinetic effects. Steroid interactions with one site underlie the increase in the fraction of long openings (OT3) and the reduction in the fraction of CTβ ("Site A" effects), and steroid activity in the second site cause the prolongation of OT3 ("Site B" effect) (Akk et al., 2004). More recent data using a benz[e]indene neurosteroid analog suggest that the Site A effects may be mediated by steroid interactions with two distinct sites (Li et al., 2006).

The results from the present work, and those from a previous study in which ethanol was used to modulate receptor interactions with a synthetic neurosteroid ACN (Akk and Steinbach, 2003), demonstrate that ethanol interactions with the GABA_A receptor enhance steroid-dependent prolongation of OT3. The 3α5αP dose-response curve for the effect on OT3 duration was shifted towards lower steroid concentrations by a factor of 100 in the presence of 0.1 % (17 mM) ethanol while no corresponding effect was seen in the two remaining kinetic parameters affected by higher concentrations of steroid. The application of ethanol alone has been shown to be without effect on single-channel currents elicited by GABA (Akk and Steinbach, 2003).

Two lines of evidence suggest that ethanol modulates steroid interactions with Site B as opposed to a novel steroid site being revealed in the presence of ethanol through which then the prolongation of OT3 is accomplished. First, 17-PA, a steroid antagonist which blocks the ability of high doses of 3α5αP to prolong OT3 duration, also reduces the ethanol-induced prolongation of OT3 by 3α5αP. Second, the replacement of the carboxy terminus of the γ2 subunit with homologous sequence from the ρ subunit, a procedure which reduces the ability of 3α5αP to prolong OT3, also reduces the ethanol-dependent prolongation of OT3. The simplest explanation of these results is that steroid interactions with the same site underlie OT3 prolongation seen in the absence and
presence of ethanol, and that the action of ethanol is to increase the affinity of
neurosteroid for Site B.

We also sought to determine whether ethanol affected the actions of two
additional endogenous steroids, pregnenolone sulfate and β-estradiol. The motivation for
these studies was two-fold. First, neither pregnenolone sulfate nor β-estradiol is thought
to act at steroid potentiating sites. Thus, prolongation of OT3 duration in the presence of
ethanol and pregnenolone sulfate or β-estradiol would indicate that steroid interactions
with a novel, nonselective site underlie the effect seen in the presence of ethanol and
steroids. Secondly, it is not known whether ethanol could enhance inhibitory actions. The
present study shows that no changes in receptor kinetic properties were observed when
pregnenolone sulfate was coapplied with ethanol. This finding supports the hypothesis
that steroid interaction with the characterized potentiating steroid site, and not a novel
hitherto unidentified site, is the key to the effect seen in the presence of ethanol.

While the coapplication of ethanol with β-estradiol did not result in further
modulation of receptor function, indicating a lack of interactions between this steroid and
Site B, receptor exposure to β-estradiol slowed the deactivation decay in whole-cell
recordings by almost two-fold. This may be a result of the increase in the fraction of OT3,
observed in single-channel recordings, or an enhanced contribution from the short-lived
desensitized state. To the best of our knowledge, this is the first study in which the kinetic
mechanism of β-estradiol action on the GABA_{A} receptor has been evaluated. It is unlikely,
however, that this finding has a physiological significance since the β-estradiol serum
concentration is typically in the nanomolar range whereas our, rather modest, effect was
observed in the presence of micromolar concentrations of steroid.

To our surprise, the clear-cut single-channel effect of ethanol on channel
modulation by 3α5αP did not translate into changes in macroscopic responses.
Coapplication of 3α5αP and ethanol, leading to a more than 2-fold increase in the duration of OT3 in single-channel records, had no effect whatsoever on the whole-cell current peak response. While we lack evidence to conclusively account for this discrepancy we can offer several possible explanations. First, the contribution of the OT3 component to the mean open duration, and consequently to channel open probability, is relatively small due to the low frequency of the event. Therefore, even large changes in its duration tend to have a small overall effect on channel open probability. Simple calculations show that with unchanged durations for OT1 and OT2, and unchanged relative frequencies for all open time components, an increase from 7.6 to 18 ms in the OT3 duration results in an approximately 20 % increase in the open probability. The effect would be slightly more pronounced at lower GABA concentrations where the mean closed time is greater and, thus, a change in the mean open time would have a larger effect, or if the relative contribution of OT3 were greater. The increase in the open probability due to a prolonged OT3 may also have been partially offset by opposing effects from changes in other kinetic parameters (e.g., an increase in the fraction of CTβ, Figure 1B), or be inconspicuous due to low overall experimental resolution.

Another potential explanation comes from the work of Feng et al. (2004) who proposed that, at least in some activation models, the peak current is determined by openings into the OT2 state. In their model, changes in the OT3 duration were predicted to have only a minor effect on peak response. Additional factors, e.g., desensitization, may mask the changes in steady state current levels due to changes in OT3 duration which in any case are predicted to be relatively small (see above).

Clearly, the present results demonstrate the need to carry out parallel experiments at microscopic and macroscopic levels. The single-channel studies provide an excellent tool for determining mechanisms of action following pharmacological or molecular...
manipulations, but should be complemented by whole-cell recordings to provide a better overall view of the results as some kinetic effects which are obvious in single-channel recordings may not be so important in the whole-cell or synaptic context. We realize that it may be argued that a physiological effect of a drug or a mixture of drugs may more closely follow the effects seen in whole-cell recordings.

We were able to replicate the core single-channel findings on mutual synergism in the actions of ethanol and $3\alpha5\alpha P$ in tadpole behavioral assays. The presence of $3\alpha5\alpha P$, at a concentration where no steroid effect could be observed, shifted the ethanol dose-response curve for loss of righting reflex to lower concentrations. The effect was likely mediated via steroid interactions with the $GABA_A$ receptor because no such shift was seen in tadpoles exposed to $3\alpha5\alpha P$ in the presence of steroid antagonist, 17-PA.

The observation that ethanol and $3\alpha5\alpha P$ interact in both behavioral and in vitro electrophysiologic assays indicates a possible causal relationship. However, the lack of data on the precise mechanisms of tadpole anesthesia precludes us from making a direct comparison to our studies on recombinant receptors.

A previous study (Mennerick et al., 2004) showed that the inhibitory effect of 17-PA is specific to $5\alpha$-reduced steroids. It will be interesting to determine in the future whether $5\beta$-reduced steroids (e.g., pregnanolone) can act similarly to $3\alpha5\alpha P$ in modifying ethanol interactions with the receptor, and whether such effect is insensitive to the steroid antagonist.

The issue of subunit composition of $GABA_A$ receptors in mammalian brain responsible for sedation or hypnosis induced by concentrations of ethanol reached in a social setting (generally, ≤30 mM) is unresolved. The importance of $\alpha4$ (or $\alpha6$) and $\delta$ subunit containing receptors, in combination with either $\beta2$ or $\beta3$ subunits, in ethanol actions has been proposed in several published works (Sundstrom-Poromaa et al., 2002;
Wallner et al., 2003). In contrast, Borghese et al (2006) and Yamashita et al (2006) found no potentiating effect by low concentrations of ethanol on receptors with such subunit compositions. Consistent with the latter finding, mice lacking the α6 subunit had ethanol-induced sleep times comparable to that in wild-type mice, while δ subunit knockout mice had mostly unaltered ethanol-induced behavioral effects (Homanics et al., 1997; Mihalek et al., 2001). In addition, the role of the α1 subunit in, at least some effects of ethanol has been conclusively shown by experiments on knockout and knockin mice (Blednov et al., 2003; Kralic et al., 2003; Werner et al., 2006). Results from the present work further support the role of α1 subunit containing receptors in ethanol-induced changes in the central nervous system.
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FOOTNOTES

JHS is the Russell and Mary Shelden Professor of Anesthesiology. Support for this work was provided by National Institutes of Health grants AA14707 and GM47969.

Address correspondence to: Gustav Akk, Department of Anesthesiology, Washington University in St Louis, Campus Box 8054, 660 S. Euclid Ave, St Louis, MO 63110; Tel: (314) 362-8565; Fax: (314) 362-8571; Email: akk@morpheus.wustl.edu
LEGENDS FOR FIGURES

Figure 1. Ethanol has a dose-dependent effect on the ability of 3α5αP to prolong OT3 duration. A. Sample single-channel currents and open and closed time histograms from the respective patches recorded in the presence of 50 µM GABA, GABA + 10 nM 3α5αP, GABA + 3α5αP + 0.02 % ethanol, or GABA + 3α5αP + 0.1 % ethanol. Channel openings are downward deflections. The presence of 0.1 but not 0.02 % ethanol resulted in significant prolongation of duration of OT3 (shown with arrow). The open times were 0.29 ms (24 %), 2.3 ms (51 %) and 4.1 ms (25 %) (GABA); 0.33 ms (37 %), 2.7 ms (52 %) and 7.9 ms (11 %) (GABA+3α5αP); 0.40 ms (29 %), 2.3 ms (45 %) and 8.5 ms (25 %) (GABA+3α5αP+0.02 % ethanol); and 0.39 ms (37 %), 3.5 ms (39 %) and 20.4 ms (24 %) (GABA+3α5αP+0.1 % ethanol). The closed times were 0.22 ms (57 %), 1.9 ms (13 %) and 9.9 ms (30 %) (GABA); 0.20 ms (47 %), 1.2 ms (32 %) and 12.0 ms (21 %) (GABA+3α5αP); 0.30 ms (53 %), 2.6 ms (29 %) and 15.2 ms (18 %) (GABA+3α5αP+0.02 % ethanol); and 0.17 ms (57 %), 1.3 ms (24 %) and 13.7 ms (18 %) (GABA+3α5αP+0.1 % ethanol). B. The relationship between ethanol concentration (in % vol/vol) and OT3 duration, relative weight of OT3, and relative weight of CTβ. The data are for receptors exposed to 50 µM GABA, 10 nM 3α5αP and varying concentrations of ethanol. Each symbol corresponds to data from one patch. The relationship between the duration of OT3 and ethanol concentration was fitted to: Y([steroid])=Y₀ + (Y_max - Y₀)/([steroid] + EC₅₀). Maximal OT3 duration was 17.6±1.4 ms and the EC₅₀ was 0.015±0.009 %. The low concentration asymptote was set to 6 ms. The relationship for relative weight of OT3 vs. ethanol concentration was fitted to linear equation resulting in a nonsignificant correlation (R²=-0.1; p=0.77). Higher concentrations of ethanol tended to increase the relative frequency of the CTβ component, however, the correlation was
Figure 2. The presence of 0.1 % (17 mM) ethanol affects the ability of $3\alpha 5\alpha P$ to prolong OT3 duration. All data were obtained using 50 µM GABA to activate the receptor. Each symbol corresponds to data from one patch. The curves were fitted using: 

$$Y([\text{steroid}])=Y_0 + \frac{(Y_{\text{max}} - Y_0) [\text{steroid}]}{[\text{steroid}] + EC_{50}}.$$ 

The data for control conditions are from Akk et al. (2005). 

A. The effect of $3\alpha 5\alpha P$ on OT3 duration is shifted to lower steroid concentrations in the presence of 0.1 % ethanol. The best-fit parameters for control conditions were: $Y_0=5.2\pm2.3$ ms, $Y_{\text{max}}=14.9\pm1.1$ ms, $EC_{50}=66.2\pm54.3$ nM, for +0.1 % ethanol: $Y_0=5.4\pm5.2$ ms, $Y_{\text{max}}=17.8\pm1.4$ ms, $EC_{50}=0.42\pm0.50$ nM. 

B. The presence of ethanol did not affect the relative weight of OT3. The best-fit parameters for the curve describing the control data were: $Y_0=0.16\pm0.08$, $Y_{\text{max}}=0.48\pm0.06$, $EC_{50}=119\pm125$ nM. No curve fitting was carried out for data obtained in the presence of ethanol because of large scatter in the data. The dashed line shows the calculated mean value for the data points obtained in the presence of ethanol. 

C. The presence of ethanol did not affect the relative weight of CT$\beta$. The best-fit parameters for the curve describing the control data were: $Y_0=0.25\pm0.04$, $Y_{\text{max}}=0.04\pm0.03$, $EC_{50}=94\pm73$ nM, for +0.1 % ethanol: $Y_0=0.19\pm0.01$, $Y_{\text{max}}=0.04\pm0.06$, $EC_{50}=113\pm154$ nM.

Figure 3. 17-PA affects prolongation of OT3 achieved by a low concentration of $3\alpha 5\alpha P$ in the presence of ethanol, or by high concentration of $3\alpha 5\alpha P$. Sample single-channel currents and open and closed time histograms in the presence of 50 µM GABA + 1 µM $3\alpha 5\alpha P$, GABA + 1 µM $3\alpha 5\alpha P$ + 10 µM 17-PA, GABA + 10 nM $3\alpha 5\alpha P$ + 0.1 % ethanol, or GABA + 10 nM $3\alpha 5\alpha P$ + 0.1 % ethanol + 17-PA. Channel openings are downward deflections. The open times were 0.35 ms (39 %), 1.3 ms (24 %) and 16.1 ms.
(36%) \((\text{GABA}+1 \mu\text{M }3\alpha 5\alpha \text{ P})\); 0.34 ms (43%), 1.9 ms (18%) and 8.5 ms (40%)

\((\text{GABA}+3\alpha 5\alpha \text{ P}+10 \mu\text{M }17\text{-PA})\); 0.39 ms (37%), 3.5 ms (39%) and 20.4 ms (24%)

\((\text{GABA}+10 \text{nM }3\alpha 5\alpha \text{ P}+0.1 \% \text{ ethanol})\); and 0.35 ms (28%), 3.8 ms (61%) and 9.9 ms (11%)

\((\text{GABA}+3\alpha 5\alpha \text{ P}+\text{ ethanol}+17\text{-PA})\). The closed times were 0.22 ms (55%), 1.1 ms (39%) and 13.5 ms (6%) \((\text{GABA}+1 \mu\text{M }3\alpha 5\alpha \text{ P})\); 0.30 ms (54%), 1.7 ms (37%) and 10.6 ms (9%)

\((\text{GABA}+3\alpha 5\alpha \text{ P}+10 \mu\text{M }17\text{-PA})\); 0.17 ms (57%), 1.3 ms (24%) and 13.7 ms (18%)

\((\text{GABA}+10 \text{nM }3\alpha 5\alpha \text{ P}+0.1 \% \text{ ethanol})\); and 0.16 ms (61%), 1.5 ms (16%) and 14.7 ms (24%)

\((\text{GABA}+3\alpha 5\alpha \text{ P}+\text{ ethanol}+17\text{-PA})\). The data for \((\text{GABA}+10 \text{nM }3\alpha 5\alpha \text{ P}+0.1 \% \text{ ethanol})\) are from Figure 1. 17-PA affects prolongation by 1 \mu\text{M }3\alpha 5\alpha \text{ P}, and 10 nM AP + ethanol similarly, suggesting that the same site on the receptor is responsible for both types of prolongation.

**Figure 4. The substitution of the C'-terminal end of the \(\gamma\) subunit with homologous sequence from the \(\rho\) subunit results in reduced ability for \(3\alpha 5\alpha \text{ P}\) to prolong the OT3 duration in the presence of ethanol.** Sample single-channel currents from the \(\alpha\beta(\gamma-\rho)\) receptor, and open and closed time histograms in the presence of 50 \mu\text{M} GABA, GABA + 0.1 % ethanol, GABA + 0.1 \mu\text{M }3\alpha 5\alpha \text{ P}, or GABA + 3\alpha 5\alpha \text{ P} + ethanol. Channel openings are downward deflections. The open times were 0.36 ms (18%), 4.7 ms (56%) and 7.0 ms (26%) \((\text{GABA})\); 0.38 ms (21%), 2.5 ms (32%) and 6.4 ms (47%) (\(\text{GABA}+\text{ ethanol})\); 0.29 ms (27%), 1.8 ms (16%) and 8.4 ms (57%) \((\text{GABA}+3\alpha 5\alpha \text{ P})\); and 0.16 ms (12%), 6.8 ms (64%) and 12.2 ms (24%) \((\text{GABA}+3\alpha 5\alpha \text{ P}+\text{ ethanol})\). The closed times were 0.13 ms (49%), 2.1 ms (32%) and 15.6 ms (18%) \((\text{GABA})\); 0.15 ms (59%), 1.4 ms (16%) and 13.2 ms (25%) \((\text{GABA}+\text{ ethanol})\); 0.19 ms (30%), 1.3 ms (30%) and 8.3 ms (40%) \((\text{GABA}+3\alpha 5\alpha \text{ P})\); and 0.11 ms (31%), 1.6 ms (32%) and 16.1 ms (38%) \((\text{GABA}+3\alpha 5\alpha \text{ P}+\text{ ethanol})\). The OT3 is shown with arrows in histograms.
Figure 5. The presence of 0.1 % (17 mM) ethanol does not show synergism with 3α5αP in macroscopic recordings. A. Macroscopic responses to 5 µM and 1 mM GABA. 5 µM GABA corresponds to EC_{25-30} in whole-cell recordings, and is thus analogous to the 50 µM GABA used in single-channel recordings. B. Exposure of the same cell to 5 µM GABA, GABA + 2 nM 3α5αP, GABA + 0.1 % ethanol, or GABA + 3α5αP + ethanol. No significant changes in peak amplitude were observed in the simultaneous presence of steroid and ethanol.

Figure 6. The presence of 1 % (170 mM) ethanol does not affect receptor modulation by pregnenolone sulfate. A. Single-channel clusters in the presence of 50 µM GABA and 25 µM pregnenolone sulfate (PS) in the absence and presence of 1 % ethanol. Channel openings are downward deflections. Putative clusters are identified with lines below the current traces. The presence of pregnenolone sulfate results in reduced cluster durations due to a faster apparent desensitization rate. Ethanol does not affect the ability of the steroid to reduce cluster durations. The summary of intracluster open and closed time distributions in the absence and presence of ethanol is given in Table 4. B. Macroscopic recordings demonstrate the absence of an effect of ethanol on peak response or desensitization time constant.

Figure 7. The presence of 1 % (170 mM) ethanol does not affect receptor modulation by 10 µM β-estradiol. A. Single-channel currents in the presence of 50 µM GABA and 10 µM β-estradiol in the absence and presence of 1 % ethanol. Channel openings are downward deflections. The presence of β-estradiol results in an increase in the relative weight of OT3 and a split of the CTβ closed time component into two
components. The open times were 0.15 ms (14 %), 1.3 ms (21 %) and 5.1 ms (65 %) (GABA+β-estradiol); and 0.47 ms (33 %), 2.1 ms (6 %) and 6.1 ms (61 %) (GABA+β-estradiol+ethanol). The closed times were 0.14 ms (59 %), 1.6 ms (11 %), 9.8 ms (23 %) and 38.5 ms (7 %) (GABA+β-estradiol); and 0.17 ms (61 %), 1.1 ms (19 %), 13.7 ms (17 %) and 33.2 ms (4 %) (GABA+β-estradiol+ethanol). B. Current traces obtained in the presence of 10 µM GABA, GABA + 1 % ethanol, GABA + 10 µM β-estradiol, or GABA + ethanol + β-estradiol. Macroscopic recordings suggest that ethanol has a minor effect on peak response or decay time constants. However, the presence of β-estradiol significantly increases the deactivation time constant both in the absence and presence of ethanol. The right panel shows a portion of the data given in the left panel at a higher time resolution.
Table 1. The summary of single-channel kinetic analysis from the wild-type receptor under control conditions and in the presence of combinations of ethanol and 3α5αP in the absence and presence of steroid antagonist, 17-PA. The mean durations (OT1-3, CT1, CT2 and CTβ) and relative contributions (fraction OT1-3, fraction CT1, CT2 and CTβ) for the three open and closed time components are shown. All data were obtained in the presence of 50 µM GABA to activate the channel. The control data (no steroid, no ethanol) are from Akk et al. (2005). Statistical analysis was carried out using ANOVA with Bonferroni correction (Systat 7.0, SSPS, Chicago, IL). For 3α5αP, the significance level applies to comparison to no steroid/no ethanol (control) condition. For 3α5αP + 17-PA, the significance levels apply to comparison to control condition, and to 3α5αP alone, respectively.

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<td>0.1 %</td>
<td>0.16±0.04</td>
<td>0.38±0.16</td>
<td>1.5±0.1</td>
<td>0.27±0.05</td>
<td>12.6±6.4</td>
<td>0.35±0.14</td>
<td>8</td>
</tr>
<tr>
<td>1 µM</td>
<td>0.1 %</td>
<td>0.16±0.03</td>
<td>0.34±0.22</td>
<td>1.6±0.1</td>
<td>0.32±0.05</td>
<td>14.4±9.8</td>
<td>0.34±0.17</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 2. The summary of single-channel kinetic analysis under control conditions and in the presence of combinations of ethanol and 3α5αP for the receptor containing a γ subunit where the carboxyterminal sequence was replaced with homologous sequence from the ρ subunit (γ–ρ subunit). The mean durations (OT1-3, CT1, CT2 and CTβ) and relative contributions (fraction OT1-3, fraction CT1, CT2 and CTβ) for the three open and closed time components are shown. All data were obtained in the presence of 50 µM GABA to activate the channel. Statistical analysis was carried out using ANOVA with Bonferroni correction (Systat 7.0, SSPS, Chicago, IL). Only the fraction of OT3 for 1 µM 3α5αP reached significant (p<0.05) difference from control (no steroid/no ethanol) conditions. All other parameters examined (mean duration OT3, fraction OT3, fraction CTβ), at all other conditions studied showed nonsignificant effects.
Table 3. Ethanol and steroid effects on tadpole righting reflexes. The loss of righting reflex in tadpoles is affected in a manner consistent with the hypothesis of synergistic effects of potentiating steroids and ethanol. 30 nM 3α5αP alone did not produce LRR in tadpoles (estimated from four experiments using a total of forty tadpoles). The values are best-fit parameters ± standard deviation estimated from a fit of the Hill equation \( Y = ([\text{ethanol}]^{n_H} / ([\text{ethanol}]^{n_H} + EC_{50}^{n_H}) \), where \( EC_{50} \) is the concentration causing half-maximal effect and \( n_H \) is the Hill coefficient. A total of 20 tadpoles were exposed to each condition, in 2 replicates of 10 tadpoles each. The difference in the concentration-effect relationships was assessed using a 2-tailed Kolmogorov-Smirnov 2 sample test, with \( N = 20 \) observations. The concentration-effect relationship for ethanol + 30 nM 3α5αP differed from that for ethanol alone (\( P < 0.05 \)), while no other comparisons showed a significant difference (\( p > 0.05 \)).

<table>
<thead>
<tr>
<th>Steroid(s)</th>
<th>Loss of righting reflex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ethanol EC(_{50}) (mM)</td>
</tr>
<tr>
<td>-</td>
<td>228.2±0.5</td>
</tr>
<tr>
<td>30 nM 3α5αP</td>
<td>107.0±2.2</td>
</tr>
<tr>
<td>30 nM 3α5αP + 10 µM 17-PA</td>
<td>244.2±23.4</td>
</tr>
<tr>
<td>10 µM 17-PA</td>
<td>221.8±18.9</td>
</tr>
</tbody>
</table>
Table 4. The summary of single-channel kinetic analysis from the wild-type receptor under control conditions and in the presence of ethanol and pregnenolone sulfate (PS) or β-estradiol. The mean durations (OT1-3, CT1, CT2 and CTβ) and relative contributions (fraction OT1-3, fraction CT1, CT2 and CTβ) for the three open and closed time components are shown. In the presence of β-estradiol, an additional closed time component was observed (both shown in the CTβ columns). All data were obtained in the presence of 50 µM GABA to activate the channel. The control data (no steroid, no ethanol) are from Akk et al. (2005). Statistical analysis was carried out using ANOVA with Bonferroni correction (Systat 7.0, SPSS, Chicago, IL). None of the parameters examined (mean duration OT3, fraction OT3, fraction CTβ) showed significant effects.
Figure 3

50 μM GABA + 1 μM 3α5αP

GABA + 1 μM 3α5αP + 10 μM 17-PA

GABA + 10 nM 3α5αP + 0.1 % ethanol

GABA + 10 nM 3α5αP + ethanol + 10 μM 17-PA

closed
open

duration (ms)
Figure 5

A

5 μM GABA

1 mM GABA

B

5 μM GABA
+2 nM 3α5αP + 0.1 % ethanol
+0.1 % ethanol
+2 nM 3α5αP

1 s

200 pA

100 pA
Figure 7

A  GABA + β-estradiol

closed

open

GABA + β-estradiol + 1% ethanol

B

sqrt(fraction of events/bin)

duration (ms)

100 ms

1 pA

1 s

200 ms

- GABA
- +ethanol
- +β-estradiol
- +ethanol+β-estradiol