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# NATURAL AND ENANTIOMERIC ETIOCHOLANOLONE INTERACT WITH DISTINCT SITES ON THE RAT $\alpha1\beta2\gamma2L$ $GABA_A$ RECEPTOR

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Running title - etiocholanolone enantioselectivity at the $GABA_A$ receptor

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**ABSTRACT**

We have studied the ability of the androgen etiocholanolone and its enantiomer (*ent*-etiocholanolone) to modulate the rat $\alpha1\beta2\gamma2L$ $GABA_A$ receptor function transiently expressed in HEK cells. Studies on steroid enantiomer pairs can yield powerful new information on the pharmacology of steroid interactions with the $GABA_A$ receptor. Both steroids enhance currents elicited by GABA but *ent*-etiocholanolone is much more powerful than etiocholanolone at producing potentiation. At a low GABA concentration (0.5 μM, $<EC_5$), the presence of 10 μM *ent*-etiocholanolone potentiates whole-cell currents by almost 30-fold while 10 μM etiocholanolone merely doubles the peak response. At higher GABA concentration (5 μM, $\sim EC_{25}$), the potentiation curve for *ent*-etiocholanolone is positioned at lower concentrations than that for etiocholanolone. Single-channel kinetic analysis shows that exposure to etiocholanolone has a single effect on currents: the relative frequency of long openings is increased in the presence of steroid. But exposure to *ent*-etiocholanolone produces two kinetic effects: an increase in the relative frequency of long openings, and a decrease in the frequency of long closed times. The presence of etiocholanolone does not inhibit potentiation by *ent*-etiocholanolone suggesting that etiocholanolone is unable to interact with the sites through which *ent*-etiocholanolone modifies receptor function. The double mutation $\alpha1$(N407A/Y410F) prevents potentiation by etiocholanolone but not by *ent*-etiocholanolone, and the $\alpha1$(Q241A) and $\alpha1$(I238N) point mutations fully abolish potentiation by etiocholanolone but not by *ent*-etiocholanolone. We conclude that etiocholanolone and its enantiomer interact with distinct sites on the $\alpha1\beta2\gamma2L$ $GABA_A$ receptor.

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## INTRODUCTION

Potentiating steroids modulate $GABA_A$ receptor activity via interactions with binding sites located within the membraneous domains of the receptor (Rick et al., 1998; Akk et al., 2005; Hosie et al., 2006). The existing electrophysiological data support three distinct, but possibly allosterically coupled, sites for steroids, each influencing a distinct kinetic aspect of channel activation. Two parameters of channel openings (the duration and fraction of long openings) and one parameter of channel closings (the fraction of the activation-related closed time) are affected upon exposure to many neuroactive steroids (Twyman and Macdonald, 1992; Akk et al., 2004). The structural basis of steroid potentiation is much less well understood. Recent data pinpoint a potentiating steroid site in a cavity between the M1 and M4 transmembrane domains in the $\alpha$ subunit where specific residues act as hydrogen bond donor and acceptor to stabilize the steroid molecule (Hosie et al., 2006). Despite this obviously important finding, it remains unresolved whether this site represents a common interaction site for potentiating steroids or whether structurally diverse steroids interact with distinct, although possibly overlapping, binding sites, and how the identified site relates to the multiple kinetic effects observed for steroids.

An enantiomer is a stereoisomer of an optically active compound in which all chiral centers have the opposite configuration resulting in a mirror image of itself. As a result, the steroids of an enantiomer pair have identical chemical and physical properties (i.e., interactions with the lipid membrane) but may differ in their ability to interact with specialized binding pockets on receptors or other targets with chiral centers. Previous work has established that the actions of many steroids on the $GABA_A$ receptor are enantioselective. For example, the enantiomers of endogenous neurosteroids allopregnanolone and pregnanolone are weaker potentiators of receptor function than the natural steroids (Covey et al., 2000). In contrast, enantiomers of androsterone and etiocholanolone are more effective at modulating the $GABA_A$ receptor function than the natural steroids (Katona et al., submitted). The enantioselectivity of steroid modulators is not limited to the $GABA_A$ receptor. In the related $GABA_C$ receptor, pregnanolone blocks receptor

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activation by GABA, but its enantiomer acts as a potentiator (Li et al., 2006b). Human nicotinic α4β2 receptors are potentiated by 17β-estradiol but not by its enantiomer (Paradiso et al., 2001). Finally, the block of T-type $Ca^{2+}$ channels by (3β,5α,17β)-17-hydroxyestrane-3-carbonitrile has been shown to be enantioselective (Todorovic et al., 1998).

Two interpretations for enantioselectivity have been put forward. First, the differences in the actions of natural and enantiomer steroids have been interpreted as rising from non-optimal interactions between a common chiral binding pocket and one of the steroids within the enantiomer pair. Another interpretation is that interactions with distinct binding sites underlie the actions of natural and enantiomer steroids, and that the ability to efficiently interact with their individual sites determines the potency and efficacy of the steroids within an enantiomer pair (Katona et al., submitted; Wittmer et al., 1996).

In this manuscript, we present results from studies on channel modulation by the androgen, etiocholanolone and its enantiomer. This pair of steroids is uncommon in that the enantiomer is a stronger potentiator of the $GABA_A$ receptor than the natural isomer. We introduce data indicating that the natural and enantiomer versions of etiocholanolone potentiate the $GABA_A$ receptor via different kinetic mechanisms, and that steroid interactions with distinct sites underlie their effects.

## MATERIALS AND METHODS

Experiments were conducted on HEK293 cells (ATCC Number CRL-1573) expressing rat α1β2γ2L $GABA_A$ receptors as described previously (Akk et al., 2001; 2004; Li et al., 2006a). The subunit cDNAs were subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA) and expressed in HEK cells using a calcium phosphate precipitation-based transient transfection technique (Akk, 2002). The α1 subunit is epitope (FLAG) tagged in the aminoterminal end of the subunit (Einhauer and Jungbauer, 2001; Ueno et al., 1996). The presence of the FLAG tag is without effect on channel kinetics (Ueno et al., 1996; unpublished observations).

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The experiments were carried out using standard single-channel patch clamp and whole-cell voltage clamp methods. The bath solution contained (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 HEPES; pH 7.4. In single-channel recordings, the pipet solution contained (in mM): 120 NaCl, 5 KCl, 10 $MgCl_2$, 0.1 $CaCl_2$, 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 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, pH 7.4.

The agonist (GABA) and steroid modulators 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. The maximal DMSO concentration in diluted steroid solutions was 0.3 %. Channel activation by GABA was not affected by the presence of 0.3 % DMSO (data not shown). All experiments were carried out at room temperature.

The recording and analysis of single-channel currents have been described in detail previously (Akk et al., 2001; 2004). All currents were obtained at 50 μM GABA, a concentration which corresponds to approximately $EC_{30}$ in the open probability dose-response curve (Steinbach and Akk, 2001). The pipet potential was held at +60 to +80 mV, which translates to an approximately -120 to -100 mV potential difference across the patch membrane. The channel activity 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 key features of the analysis of single-channel currents are the following. The analysis 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 open and closed times were estimated from the idealized currents using a maximum likelihood method which incorporates a correction for missed events (QuB Suite;

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www.qub.buffalo.edu).

The recording and analysis of whole-cell currents was carried out as described previously (Li et al., 2006a). The cells were clamped at -60 mV. The cells were exposed to GABA and steroids for 4 s with 30 s washouts separating successive applications. The current traces were low-pass filtered at 2 kHz and digitized at 10 kHz. The analysis of whole-cell currents was carried out using the pClamp 9.0 software package, and was aimed at determining the peak amplitude.

The enantiomer of etiocholanolone was synthesized as described in Katona et al. (submitted). Etiocholanolone, 3$\alpha$5$\beta$P and other chemicals were purchased from Sigma Chemical Co (St Louis, MO).



## RESULTS

### Different kinetic actions underly the ability of etiocholanolone and its enantiomer to potentiate the $GABA_A$ receptor

While both natural and *ent*-etiocholanolone can potentiate receptor activity elicited by GABA, the enantiomer is much stronger at doing so than its natural counterpart. When coapplied with 0.5 μM GABA (<$EC_5$, Li et al., 2006a), 10 μM *ent*-etiocholanolone results in 28±10-fold potentiation of whole-cell response compared to a 2.3±0.9-fold potentiation when 10 μM etiocholanolone is applied with GABA (n=6 cells). When the steroids are applied in the presence of 5 μM GABA (~$EC_{25}$), the potentiation dose-response curve for *ent*-etiocholanolone is shifted to lower steroid concentrations compared to the dose-response curve for etiocholanolone (Figure 1).

Single-channel recordings were carried out in the presence of natural and *ent*-etiocholanolone in order to determine the kinetic modes of action of the steroids. Our previous studies have shown that exposure to potentiating neuroactive steroids can result in three distinct kinetic effects on $GABA_A$ receptor currents (Akk et al., 2004; 2005). First, the relative frequency of the activation-related closed time component (i.e., channel closing rate) is decreased. Second, the

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relative frequency of the longest-lived open time component is increased. Third, the duration of long openings is increased when steroids are coapplied with GABA. Combined, the three kinetic effects contribute to potentiation observed in whole-cell recordings. Previous studies (Akk et al., 2004; Li et al., 2006a) have also shown that the number of kinetic parameters modified by a given steroid analogue varies, i.e., not all steroid analogues affect all three kinetic parameters, and that a steroid can exert the effects with different potencies. This suggests that the three kinetic effects are mediated by steroid interactions with three distinct sites, called Sites A1, A2 and B, respectively.

Sample currents recorded at 50 μM GABA, in the absence and presence of 10 μM etiocholanolone or *ent*-etiocholanolone are shown in Figure 2, and the summary of open and closed time analysis is given in Tables 1-2. The results demonstrate that coapplication of etiocholanolone with GABA leads to an increase in the relative frequency of long openings (fraction of OT3, Site A2 effect). However, in contrast to many previously studied neuroactive steroids, etiocholanolone was ineffective at increasing the duration of long openings (Site B effect) or at decreasing the relative frequency of the activation-related closed time component (Site A1 effect). When *ent*-etiocholanolone was applied with GABA, the fraction of OT3 (Site A2 effect) and the fraction of CT3 (Site A1 effect) were affected, but the steroid was relatively ineffective at increasing the duration of OT3.

Thus, *ent*-etiocholanolone possesses two of the three kinetic actions characteristic of potentiating steroids, while etiocholanolone has a single kinetic action to potentiate $GABA_A$ receptor function. Combination of the single-channel findings with the observations on the relative magnitude of potentiation seen in whole-cell recordings at low [GABA] suggests that steroid interaction with Site A1 contributes more to the cumulative whole-cell potentiation than steroid interaction with Site A2.

**Etiocholanolone does not interact with steroid Sites A1 and B**

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We next tested the ability of etiocholanolone to interact with the sites mediating a decrease in the fraction of CT3 (Site A1) and an increase in the duration of OT3 (Site B) by examining whether its presence diminishes the ability of pregnanolone (3α5βP) to potentiate $GABA_A$ receptor response. The single-channel features of potentiation by 3α5βP exhibit the full set of characteristics of potentiating steroids: increase in the duration and relative frequency of OT3 and a decrease in the frequency of CT3 (Figure 3A). By examining if and how etiocholanolone influences the single-channel open and closed time distributions for currents recorded in the presence of 3α5βP, we could test the ability of etiocholanolone to interact with additional steroid sites. A single concentration of 200 nM 3α5βP was selected for these studies. This value is at or slightly above a concentration which produces a half-maximal effect in the kinetic actions of interest (duration of OT3 and fraction of CT3), and should thus allow easy detection of competitive inhibition by etiocholanolone.

A summary of open and closed time parameters in the presence of 3α5βP in the absence and presence of 10 μM etiocholanolone is given in Tables 1-2. Data from five patches demonstrate that the addition of etiocholanolone does not influence receptor potentiation by 3α5βP. Indeed, the single-channel currents from receptors activated by 50 μM GABA in the presence of 200 nM 3α5βP and 10 μM etiocholanolone were almost identical in every aspect to currents obtained in the presence of GABA and 3α5βP but in the absence of etiocholanolone. In particular the lack of effect of etiocholanolone on the OT3 duration and fraction CT3 indicates that this steroid is unable to compete with 3α5βP for the respective sites. The sample currents are shown in Figure 3B and the summary of the results is given in Tables 1-2.

**Different binding sites underlie actions of etiocholanolone and *ent*-etiocholanolone**

A cavity within the α subunit that is lined by the N407/Y410 residues in its extracellular side has been proposed to constitute a binding pocket for steroids (Hosie et al., 2006). The two

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residues have been suggested to interact with the D-ring of a steroid molecule, and mutations to these residues reduce potentiation by many steroids as well as a related tricyclic benz[*e*]indene steroid analogue (Hosie et al., 2006; Li et al., 2006a). We examined the effects of α1(N407A/Y410F) mutations on receptor potentiation by etiocholanolone and *ent*-etiocholanolone. Sample macroscopic recordings and the steroid dose-response curves are given in Figure 4. The data indicate that the α1(N407A/Y410F) double mutation fully abolishes potentiation by the natural isomer at concentrations up to 30 μM. In contrast, potentiation by the enantiomer is shifted toward higher steroid concentrations but the efficacy of *ent*-etiocholanolone is unaffected by the double mutation. At face value, the simplest interpretation of the findings is that the binding site(s) for *ent*-etiocholanolone differ from the site for etiocholanolone, and that the α1(N407A/Y410F) double mutation most strongly affects the site through which etiocholanolone interacts with the receptor.

The data from single-channel recordings showed that *ent*-etiocholanolone and etiocholanolone interact with different classes of binding sites: *ent*-etiocholanolone influences kinetic effects mediated by Sites A1 and A2 while potentiation in the presence of etiocholanolone is achieved through steroid interactions with a single site (Site A2). Hence, an alternative explanation of the findings is that the α1(N407A/Y410F) double mutation affects only steroid interactions with Site A2 (fraction of OT3) and not those mediated by steroid interactions with Site A1. If so, then the double mutation can be expected to have a weaker effect on receptor potentiation by *ent*-etiocholanolone which utilizes the Site A1 pathway (fraction of CT3) in addition to the Site A2 pathway to potentiate receptor function, than on receptor potentiation by etiocholanolone which utilizes only the Site A2 pathway. This interpretation would be valid irrespective of whether Sites A1 and A2 are the same for etiocholanolone and *ent*-etiocholanolone.

To test this hypothesis, we examined the effect of the α1(N407A/Y410F) double mutation on channel potentiation by 3α5βP. In single-channel recordings, this steroid has been shown to possess all three kinetic effects on $GABA_A$ receptor activation (see above). Thus, a full exclusion

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of potentiation in the mutant receptor would imply that the double mutation is able to remove potentiation mediated by all three sites.

Sample currents and 3α5βP dose-response curves for wild-type and mutant receptors are shown in Figure 5. The results demonstrate that the double mutation fully eliminates channel potentiation by 3α5βP demonstrating that the mutation is able to block steroid effects via all three sites. Conversely, the finding implies that the α1(N407A/Y410F) double mutation does not affect the interactions between *ent*-etiocholanolone and either Site A1 or Site A2, and that Site A2 for etiocholanolone is distinct from Site A2 for *ent*-etiocholanolone.

The hydroxyl group at the other end of the steroid molecule (A-ring) has been suggested to interact with the Q241 residue in the α1 subunit (Hosie et al., 2006). We decided to test if a mutation to this site also differentially affects potentiation by etiocholanolone vs. *ent*-etiocholanolone. Macroscopic recordings were carried out at 5 μM GABA (~$EC_5$, dose-response data not shown) in the absence and presence of 10 μM etiocholanolone or *ent*-etiocholanolone. Sample recordings and the summary of findings are given in Figure 6. The data demonstrate that the mutation fully abolishes modulation by etiocholanolone, while the enantiomer of etiocholanolone retains some ability to potentiate the current response. Application of 10 μM *ent*-etiocholanolone potentiated the peak current to 160±24 % of control (n=10 cells, $p<0.001$) while in the presence of etiocholanolone, the peak response was 112±13 % of control (n=5 cells, $p>0.1$).

We examined the effect of one other mutation on channel potentiation by the steroid pair. The residue α1I238 has been proposed to line the steroid potentiation site, and substitution of the hydrophobic isoleucine with a polar asparagine has been shown to reduce steroid potency, presumably due to electrostatic repulsion (Hosie et al., 2006). Macroscopic recordings were carried out at 0.2 μM GABA (~$EC_5$, dose-response data not shown) in the absence and presence of 10 μM etiocholanolone or *ent*-etiocholanolone. Sample recordings and the summary of findings are given in Figure 6. The data demonstrate that this mutation also differentially affects modulation

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by etiocholanolone and *ent*-etiocholanolone. Application of 10 μM *ent*-etiocholanolone potentiated the peak current to 130±15 % of control (n=5 cells, $p<0.05$) while in the presence of etiocholanolone, the peak response was 101±9 % of control (n=5 cells, $p>0.8$).

**The natural isomer of etiocholanolone does not interact with the binding sites for *ent*-etiocholanolone**

The experiments described above were aimed at elucidating whether etiocholanolone can interact with other sites through which potentiation by natural steroids is accomplished (it cannot), and whether etiocholanolone and *ent*-etiocholanolone potentiate the receptor via the same set of sites on the receptor (they do not). We will now address the question of whether etiocholanolone can interact with the site through which *ent*-etiocholanolone acts on the frequency of CT3 (Site A1). To test that, we have examined if and how the presence of etiocholanolone affects channel potentiation by *ent*-etiocholanolone.

Whole-cell recordings were carried out in the presence of 0.5 μM GABA and 0.3, 1 or 10 μM *ent*-etiocholanolone in the absence and presence of 10 μM etiocholanolone. Sample currents are shown in Figure 7A. Exposure to 0.3, 1 or 10 μM *ent*-etiocholanolone alone resulted in 4.6±1.2 (n=7 cells), 11.0±4.8 (8 cells), or 28±10 fold (6 cells) potentiation, respectively. In the presence of 10 μM etiocholanolone, channel potentiation was 4.2±1.6, 10.6±5.3, or 28±9 fold for 0.3, 1 or 10 μM *ent*-etiocholanolone, respectively. Similarly, no effect of etiocholanolone was observed on potentiation of currents elicited by 5 μM GABA in the presence of *ent*-etiocholanolone (Figure 7B). Thus, the macroscopic data show that etiocholanolone does not inhibit potentiation by *ent*-etiocholanolone suggesting that etiocholanolone is unable to inhibit the binding of *ent*-etiocholanolone to Site A1.

We carried out analogous experiments using single-channel patch clamp to verify the absence of effect of etiocholanolone on changes in fraction of CT3. Sample single-channel

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currents obtained in the simultaneous presence of both steroids are shown in Figure 7C, and the summary of findings is presented in Tables 1-2. The results demonstrate that the presence of etiocholanolone does not impair the ability of *ent*-etiocholanolone to reduce the fraction of CT3. In addition, the relatively minor increase in the duration of OT3 (Site B effect) observed in the presence of *ent*-etiocholanolone remains unchanged when the natural isomer is coapplied with *ent*-etiocholanolone, thus agreeing with the results from whole-cell experiments and corroborating our initial finding that etiocholanolone does not interact with the sites through which *ent*-etiocholanolone acts on the receptor.



## DISCUSSION

The identification of steroid binding sites in the $GABA_A$ receptor has been a protracted process. The consensus concerning the steroid binding sites at the present time is that the binding sites are located within the membrane-spanning domains of the receptor (Rick et al., 1998; Akk et al., 2005). Recent work has identified amino acid residues within the first and fourth transmembrane domains of the $\alpha$ subunit that may act as hydrogen bond acceptor and donor, respectively, in stabilizing the binding of a steroid molecule (Hosie et al., 2006).

Despite these undoubtedly important findings, many key issues concerning $GABA_A$ receptor modulation by steroids remain obscure. The results from single-channel kinetic analysis suggest that steroid interactions with three separate interaction sites underlie channel potentiation (Li et al., 2006a). Inhibition curves of *t*-butylbicyclophosphorothionate (TBPS) binding in the presence of steroids, similarly indicate the presence of at least two interaction sites for steroids (Hawkinson et al., 1994; A. Evers, personal communication). Together, the data indicate that other potentiating sites, in addition to the one identified structurally so far, must be present on the receptor. The ability of enantiomers of natural steroids to modulate receptor function further suggests that the receptor possesses additional steroid binding sites.

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In this study, we have examined the ability of an enantiomer pair, etiocholanolone and *ent*-etiocholanolone, to potentiate $\alpha1\beta2\gamma2L$ $GABA_A$ receptor function. The effects of the steroids on GABA-mediated activation were examined individually, or in combination with each other or additional steroids, and the ability of mutations to the transmembrane domains to block potentiation by these steroids was investigated. The major finding is that etiocholanolone and *ent*-etiocholanolone act via different kinetic mechanisms to potentiate the receptor function, and that the binding sites involved in mediating potentiation are distinct.

Previous single-channel recordings have shown that exposure to many steroids results in three distinct kinetic effects, which together contribute to cumulative potentiation observed in whole-cell recordings. Such work has similarly suggested that the three kinetic effects are mediated by steroid interactions with distinct sites. Thus, the application of allopregnanolone (Akk et al., 2005) or 3α5βP (Tables 1-2) decreases the frequency of the activation-related closed time component in records (fraction of CT3, Site A1), increases the frequency of long openings (fraction of OT3, Site A2) and increases the mean duration of long openings (duration of OT3, Site B). The data presented in this manuscript demonstrate that channel potentiation in the presence of etiocholanolone is accomplished solely via the Site A2 mechanism. The finding that etiocholanolone is unable to inhibit the ability of 3α5βP to act on the duration of OT3 and fraction of CT3 suggests that etiocholanolone does not interact with sites mediating these actions, further supporting the notion that the receptor contains multiple binding sites for steroids, each responsible for a specific kinetic effect.

From a kinetic viewpoint, etiocholanolone potentiates the receptor via the Site A2 mechanism, while *ent*-etiocholanolone additionally acts via the Site A1 mechanism, and 3α5βP modulates channel activity via all three (Sites A1, A2, and B) kinetic mechanisms. Molecular manipulations in the M4 transmembrane domain (α1(N407A/Y410F) double mutation) led to full blockade of potentiation by natural steroids etiocholanolone and 3α5βP. In contrast, maximal

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potentiation by *ent*-etiocholanolone remained unaffected, although the midpoint of the dose-response curve was shifted by the double mutation. These results are inconsistent with the idea that the same sites, in terms of structure, underlie modulation by natural steroids and *ent*-etiocholanolone because any manipulation that abolishes potentiation by 3α5βP should also block potentiation by *ent*-etiocholanolone. Instead, the results suggest that Sites A1 and A2 differ, in terms of structure, for 3α5βP and *ent*-etiocholanolone, although steroid interactions with either set of sites can produce kinetically indistinguishable effects.

Two matters deserve further discussion and, possibly, follow up experiments in the future. First, the α1(Q241A) and α1(I238N) mutations, although fully blocking potentiation by etiocholanolone, also strongly reduced modulation by *ent*-etiocholanolone. Whether the mutations block one kinetic pathway fully leaving the other intact, or affect both kinetic pathways partially is unclear. A previous study (Hosie et al., 2006) suggested that the 241 site participates in signal transduction as well as steroid binding, thus suggesting that some reduction in steroid potentiation is to be expected irrespective of whether the drugs bind at the same site or not, as long as the steroids utilize the same signal transduction pathway. Similar to the double mutant, the expression levels of the transiently expressed α1(Q241A) containing receptor are low, precluding mechanistic single-channel studies.

Second, although several lines of evidence suggest multiple steroid binding sites on the $GABA_A$ receptor (see above), mutations to a single nexus, i.e., the α1(N407A/Y410F) double mutation, can block potentiation by a steroid which has a single effect (etiocholanolone, Site A2), as well as a steroid which has multiple kinetic effects (3α5βP, Sites A1, A2 and B). This finding is counterintuitive, and may suggest that the site defined by the 407/410 residues controls steroid access to multiple sites, or that steroid binding to this site allosterically controls steroid actions in other sites. In future studies it will also be important to test whether the steroid binding sites

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formed by the two $\alpha$ subunits within a receptor are equivalent, and if steroid binding to either site has the same functional effect.

Etiocholanolone has anticonvulsant activity in several seizure models in mice (Kaminski et al., 2005), and in men, low levels of androgens including etiocholanolone have been correlated with temporal lobe epilepsy (Herzog et al., 1986). However, treatment in the form of etiocholanolone injection is counterproductive because it induces the release of interleukin-1, an endogenous pyrogen in humans resulting in inflammation and fever (Watters et al., 1985). Therefore, studies on enantiomeric steroids may also help to provide a more selective, and thus clinically useful drug with the desirable GABAergic actions of etiocholanolone in the central nervous system but devoid of immunological side effects.



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**FOOTNOTES**

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## FIGURE LEGENDS

**Figure 1. Enantiomer of etiocholanolone is a stronger potentiator of the α1β2γ2L $GABA_A$ receptor than the natural form of etiochalonolone. A.** Structures of natural etiocholanolone (etiocholanolone) and enantiomer of etiocholanolone (*ent*-etiocholanolone). **B.** Etiocholanolone and *ent*-etiocholanolone potentiate currents elicited by 0.5 μM GABA (~$EC_5$). **C.** Etiocholanolone and *ent*-etiocholanolone potentiate currents elicited by 5 μM GABA (~$EC_{25}$). **D.** Steroid dose-response curves for receptors activated by 5 μM GABA. The curves were fitted to: $Y([steroid])=Y_0 + (Y_{max} - Y_0)$ [steroid]/([steroid] + $EC_{50}$). The best-fit parameters for etiocholanolone were: $Y_0$=100 % (constrained), $Y_{max}$=232±26 %, $EC_{50}$=3.1±2.2 μM. The best-fit parameters for *ent*-etiocholanolone were: $Y_0$=100 % (constrained), $Y_{max}$=282±7 %, $EC_{50}$=969±171 nM. The maximal steroid effect at 5 μM GABA is lower than that at 0.5 μM GABA because of limitation in channel maximal open probability. The data points show mean ± SEM from 3-11 cells.

**Figure 2. Etiocholanolone and *ent*-etiocholanolone potentiate the receptor via different sets of kinetic mechanisms. A.** Sample single-channel cluster from receptor activated by 50 μM GABA. Channel openings are downward deflections. The open and closed time histograms contain three components. The open times were: 0.47 ms (25 %), 3.7 ms (53 %) and 6.4 ms (22 %). The closed times were: 0.20 ms (46 %), 2.8 ms (31 %) and 12.0 ms (23 %). **B.** Sample single-channel cluster from receptor exposed to 50 μM GABA + 10 μM etiocholanolone. The open times were: 0.31 ms (18 %), 1.6 ms (13 %) and 5.7 ms (68 %). The closed times were: 0.15 ms (49 %), 1.8 ms (28 %) and 15.6 ms (23 %). The presence of steroid increased the fraction of the longest-lived open time component (shown with arrow) but did not affect other kinetic parameters typically affected by steroids (duration of the longest-lived open time component and fraction of the longest-lived closed time component). **C.** Sample single-channel cluster from receptor exposed to 50 μM

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GABA + 10 μM *ent*-etiocholanolone. The open times were: 0.39 ms (62 %), 5.2 ms (27 %) and 11.6 ms (51 %). The closed times were: 0.15 ms (62 %), 2.8 ms (28 %) and 23.3 ms (10 %). The presence of steroid increased the fraction of the longest-lived open time component and decreased the fraction of the longest closed time component (shown with arrows) but had only a minor effect at prolonging the duration of the longest-lived open time component.

**Figure 3. Channel potentiation by 3α5βP is not reduced in the presence of etiocholanolone.** **A.** The effect of 3α5βP on the duration of OT3 (left panel), fraction of OT3 (middle panel) and fraction of CT3 (right panel). The receptors were activated by 50 μM GABA in the presence of varying concentrations of steroid. Each symbol (open circle) corresponds to data from one patch. The curves were fitted to: $Y([steroid])=Y_0 + (Y_{max} - Y_0)\ [steroid]/([steroid] + EC_{50})$. The best-fit parameters for the duration of OT3 were: $Y_0$=7.0±2.3 ms, $Y_{max}$=17.9±1.8 ms, $EC_{50}$=177±162 nM. The best-fit parameters for the fraction of OT3 were: $Y_0$=0.19±0.12, $Y_{max}$=0.36±0.03, $EC_{50}$=41±85 nM. The best-fit parameters for the fraction of CT3 were: $Y_0$=0.30±0.03, $Y_{max}$=0.05±0.01, $EC_{50}$=82±35 nM. Filled squares depict data obtained in the presence of 200 nM 3α5βP and 10 μM etiocholanolone. Dotted lines show the values for duration and fraction of OT3, and fraction of CT3 for 10 μM etiocholanolone. The data demonstrate that 10 μM etiocholanolone is unable to inhibit the ability of 3α5βP to increase the duration of OT3 and decrease the fraction of CT3. **B.** Sample single-channel clusters and corresponding open and closed time histograms for receptors activated by 50 μM GABA, and exposed to 200 nM 3α5βP, or 3α5βP + 10 μM etiocholanolone. Channel openings are downward deflections. The open times were 0.45 ms (26 %), 3.2 ms (32 %) and 13.5 ms (42 %) (GABA+3α5βP); and 0.44 ms (34 %), 5.4 ms (25 %) and 20.0 ms (41 %) (GABA+3α5βP+etiocholanolone). The closed times were 0.14 ms (62 %), 1.7 ms (27 %) and 9.6 ms (12 %) (GABA+3α5βP); and 0.16 ms (61 %), 1.1 ms (27 %) and 22.2 ms (12 %) (GABA+3α5βP+etiocholanolone).

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**Figure 4. The α1(N407A/Y410F) double mutation prevents potentiation by etiocholanolone but not by *ent*-etiocholanolone. A.** Macroscopic current traces from a cell expressing α1(N407A/Y410F)β2γ2L receptors activated by 10 μM GABA (~$EC_{25}$) in the absence and presence of 10 μM etiocholanolone. The presence of steroid had a negligible effect on peak response. **B.** Macroscopic currents from mutant receptors activated by 10 μM GABA in the absence and presence of 10 μM *ent*-etiocholanolone. The presence of steroid resulted in strong potentiation. Note that macroscopic peak currents from cells expressing mutant receptors are typically several-fold smaller than those from cells expressing wild-type receptors (e.g., Figure 1). Cell surface ELISA experiments (data not shown) suggest that this is due to lower expression or trafficking, not due to a lower gating efficacy. As a result, low levels of surface receptors preclude single-channel recordings from mutant receptors. **C.** Steroid dose-response relationships for mutant receptors activated by 10 μM GABA. The curve for *ent*-etiocholanolone was fitted to: $Y([steroid])=Y_0 + (Y_{max} - Y_0)\ [steroid]/([steroid] + EC_{50})$. The best-fit parameters were: $Y_0$=100 % (constrained), $Y_{max}$=640±38 %, $EC_{50}$=5.2±1.1 μM. The double mutation fully blocks potentiation by etiocholanolone and shifts the dose-response curve for *ent*-etiocholanolone to higher steroid concentrations.

**Figure 5. The α1(N407A/Y410)F double mutation prevents potentiation by 3α5βP. A.** Macroscopic current traces from a cell expressing wild-type receptors activated by 5 μM GABA (~$EC_{25}$) in the absence and presence of 100 nM or 3 μM 3α5βP. The presence of steroid resulted in channel potentiation. **B.** Macroscopic current traces from a cell expressing α1(N407A/Y410F)β2γ2L receptors activated by 10 μM GABA (~$EC_{25}$) in the absence and presence of 100 nM or 3 μM 3α5βP. The double mutation prevented potentiation by the steroid. **C.** Wild-type and mutant receptor steroid concentration-potentiation relationships. Steroid dose-

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response curve for wild-type receptor was fitted to: $Y([steroid])=Y_0 + (Y_{max} - Y_0)$ [steroid]/([steroid] + $EC_{50}$). The best-fit parameters were: $Y_0$=100 % (constrained), $Y_{max}$=268±11 %, $EC_{50}$=330±71 nM.

**Figure 6. The α1(Q241A) and α1(I238N) mutations fully abolish channel potentiation by etiocholanolone. A.** Macroscopic current traces from α1(Q241A)β2γ2L receptors exposed to 5 μM GABA (~$EC_5$), and GABA + 10 μM etiocholanolone (top traces), or 5 μM GABA, and GABA + 10 μM *ent*-etiocholanolone (bottom traces). **B.** Macroscopic current traces from α1(I238N)β2γ2L receptors exposed to 0.2 μM GABA (~$EC_5$), and GABA + 10 μM etiocholanolone (top traces), or 0.2 μM GABA, and GABA + 10 μM *ent*-etiocholanolone (bottom traces). **C.** Summary of electrophysiological findings. Exposure to *ent*-etiocholanolone but not etiocholanolone results in channel potentiation. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (paired t-test).

**Figure 7. Etiocholanolone does not prevent *ent*-etiocholanolone binding to Site A1. A.** Macroscopic current traces from wild-type receptors exposed to 0.5 μM GABA, GABA + 10 μM etiocholanolone, GABA + 10 μM *ent*-etiocholanolone, or GABA + *ent*-etiocholanolone + etiocholanolone. The presence of etiocholanolone did not affect the current level for GABA + *ent*-etiocholanolone. **B.** Macroscopic current traces from receptors exposed to 5 μM GABA, GABA + 3 μM etiocholanolone, GABA + 3 μM *ent*-etiocholanolone, or GABA + *ent*-etiocholanolone + etiocholanolone. The presence of etiocholanolone did not affect the current level for GABA + *ent*-etiocholanolone. The data traces with the exception of combination of steroids are the same as shown in Figure 1C. **C.** Single-channel clusters from receptor exposed to 50 μM GABA + 10 μM etiocholanolone (top trace), GABA + 10 μM *ent*-etiocholanolone (middle trace), or GABA + *ent*-etiocholanolone + etiocholanolone (bottom trace). Channel openings are downward deflections. The open and closed time histograms from the respective patches are shown next to the current traces. The top and middle traces, and the respective histograms are from Figure 2. The open

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times for GABA + *ent*-etiocholanolone + etiocholanolone were: 0.28 ms (18 %), 2.6 ms (22 %) and 14.7 ms (59 %). The closed times for GABA + *ent*-etiocholanolone + etiocholanolone were: 0.14 ms (68 %), 1.4 ms (24 %) and 13.7 ms (9 %). The presence of etiocholanolone did not affect changes in the open and closed time parameters produced by *ent*-etiocholanolone.

| Steroid(s) | OT1 (ms) | Fraction OT1 | OT2 (ms) | Fraction OT2 | OT3 (ms) | Fraction OT3 | n |
|---|---|---|---|---|---|---|---|
| - | 0.28±0.07 | 0.24±0.04 | 3.1±0.8 | 0.58±0.10 | 7.6±3.0 | 0.19±0.13 | 8 |
| 10 μM etiocholanolone | 0.23±0.04 | 0.26±0.10 | 1.6±0.3 | 0.25±0.08 | 6.7±1.3$^{\dagger}$ | 0.49±0.10$^{***}$ | 7 |
| 10 μM *ent*-etiocholanolone | 0.28±0.12 | 0.20±0.05 | 2.8±1.8 | 0.23±0.05 | 11.5±3.2$^{\dagger}$ | 0.58±0.07$^{***}$ | 6 |
| 10 μM etiocholanolone + 10 μM *ent*-etiocholanolone | 0.43±0.14 | 0.25±0.11 | 4.6±1.7 | 0.31±0.13 | 15.2±3.4$^{**,\dagger}$ | 0.39±0.12$^{*,\dagger}$ | 5 |
| 200 nM 3α5βP | 0.36±0.07 | 0.30±0.07 | 2.5±0.7 | 0.28±0.04 | 11.3±2.7$^{\dagger}$ | 0.41±0.04$^{*}$ | 4 |
| 10 μM etiocholanolone + 200 nM 3α5βP | 0.38±0.06 | 0.26±0.12 | 4.9±1.7 | 0.30±0.11 | 14.7±4.4$^{**,\dagger}$ | 0.44±0.05$^{**,\dagger}$ | 4 |

**Table 1. The summary of single-channel kinetic analysis from the wild-type receptor under control conditions and in the presence of combinations of steroids.** The mean durations (OT1-3) and relative contributions (fraction OT1-3) for the three open time components are shown. All data were obtained in the presence of 50 μM GABA to activate the channel. The control data (no steroids) are from Akk et al. (2005). Statistical analysis was carried out using ANOVA with Bonferroni correction (Systat 7.0; Systat Software, Inc., Point Richmond, CA). For etiocholanolone, *ent*-etiocholanolone and 3α5βP, the significance level applies to comparison to no steroid (control) condition. For etiocholanolone + *ent*-etiocholanolone, the significance levels apply to comparison to control condition and to *ent*-etiocholanolone alone. For etiocholanolone + 3α5βP, the significance levels apply to comparison to control condition and to 3α5βP alone. $^{*}$ $P < 0.05$, $^{**}$ $P < 0.01$, $^{***}$ $P < 0.001$, $^{\dagger}$ Not significant.

| Steroid(s) | CT1 (ms) | Fraction CT1 | CT2 (ms) | Fraction CT2 | CT3 (ms) | Fraction CT3 | n |
|---|---|---|---|---|---|---|---|
| - | 0.17±0.02 | 0.57±0.06 | 1.7±0.7 | 0.14±0.05 | 13.5±5.3 | 0.29±0.02 | 8 |
| 10 μM etiocholanolone | 0.17±0.02 | 0.51±0.07 | 2.1±0.7 | 0.23±0.07 | 16.0±3.5 | 0.26±0.05$^{\dagger}$ | 6 |
| 10 μM *ent*-etiocholanolone | 0.15±0.01 | 0.59±0.09 | 1.7±0.6 | 0.28±0.03 | 17.7±6.3 | 0.13±0.08$^{***}$ | 6 |
| 10 μM etiocholanolone + 10 μM *ent*-etiocholanolone | 0.15±0.04 | 0.68±0.05 | 1.3±0.4 | 0.25±0.06 | 14.0±7.0 | 0.07±0.01$^{***,\dagger}$ | 5 |
| 200 nM 3α5βP | 0.16±0.02 | 0.60±0.04 | 1.5±0.3 | 0.29±0.02 | 8.7±1.6 | 0.11±0.04$^{***}$ | 4 |
| 10 μM etiocholanolone + 200 nM 3α5βP | 0.15±0.02 | 0.55±0.10 | 1.6±0.4 | 0.31±0.06 | 16.3±4.6 | 0.14±0.09$^{***,\dagger}$ | 4 |

**Table 2. The summary of single-channel kinetic analysis from the wild-type receptor under control conditions and in the presence of combinations of steroids.** The mean durations (CT1-3) and relative contributions (fraction CT1-3) for the three closed time components are shown. All data were obtained in the presence of 50 μM GABA to activate the channel. The control data (no steroids) are from Akk et al. (2005). Statistical analysis was carried out using ANOVA with Bonferroni correction (Systat 7.0; Systat Software, Inc., Point Richmond, CA). For etiocholanolone, *ent*-etiocholanolone and 3α5βP, the significance level applies to comparison to no steroid (control) condition. For etiocholanolone + *ent*-etiocholanolone, the significance levels apply to comparison to control condition and to *ent*-etiocholanolone alone. For etiocholanolone + 3α5βP, the significance levels apply to comparison to control condition and to 3α5βP alone. $^{*}$ $P < 0.05$, $^{**}$ $P < 0.01$, $^{***}$ $P < 0.001$, $^{\dagger}$ Not significant.

FIGURE 1

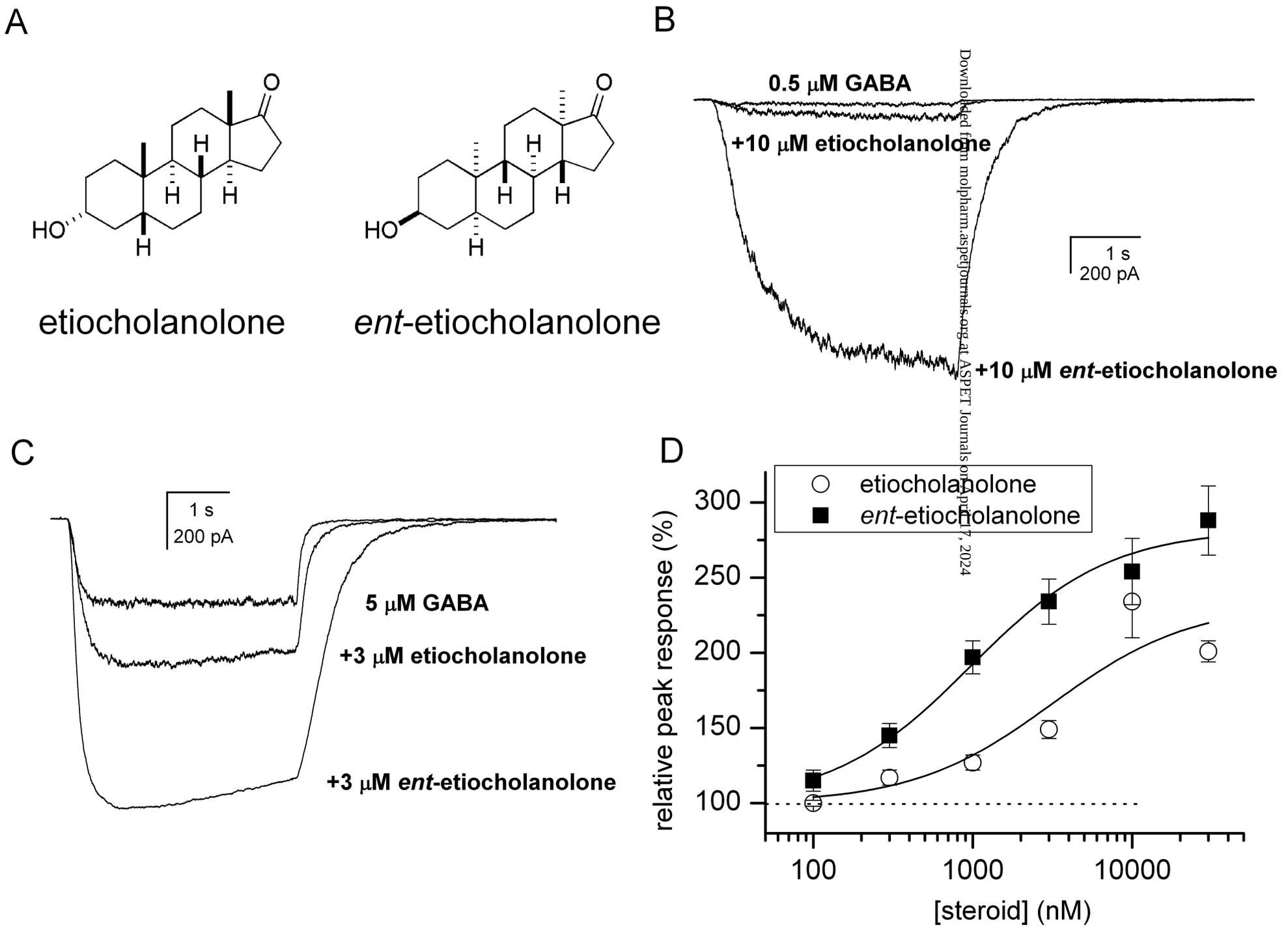

FIGURE 2

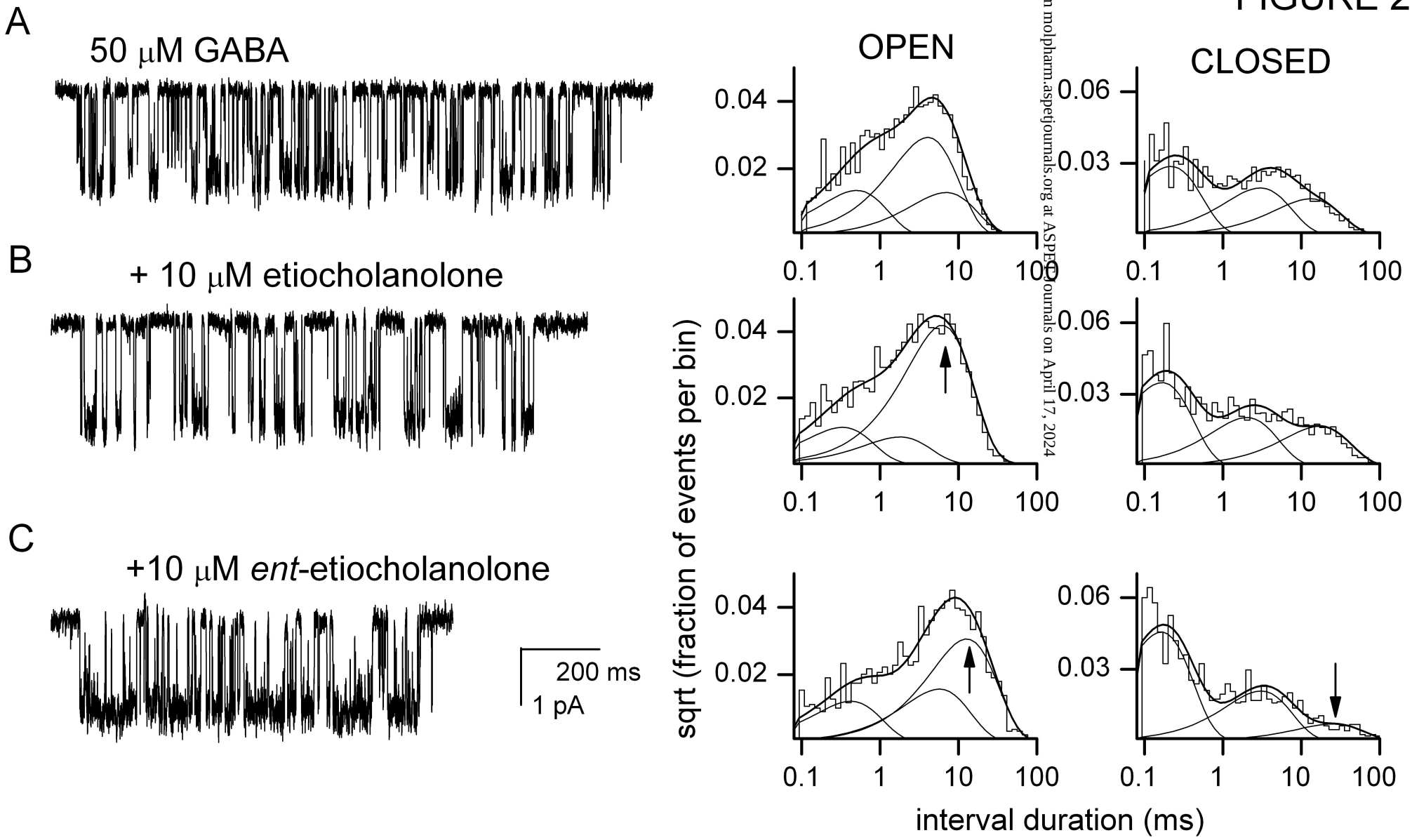

FIGURE 3

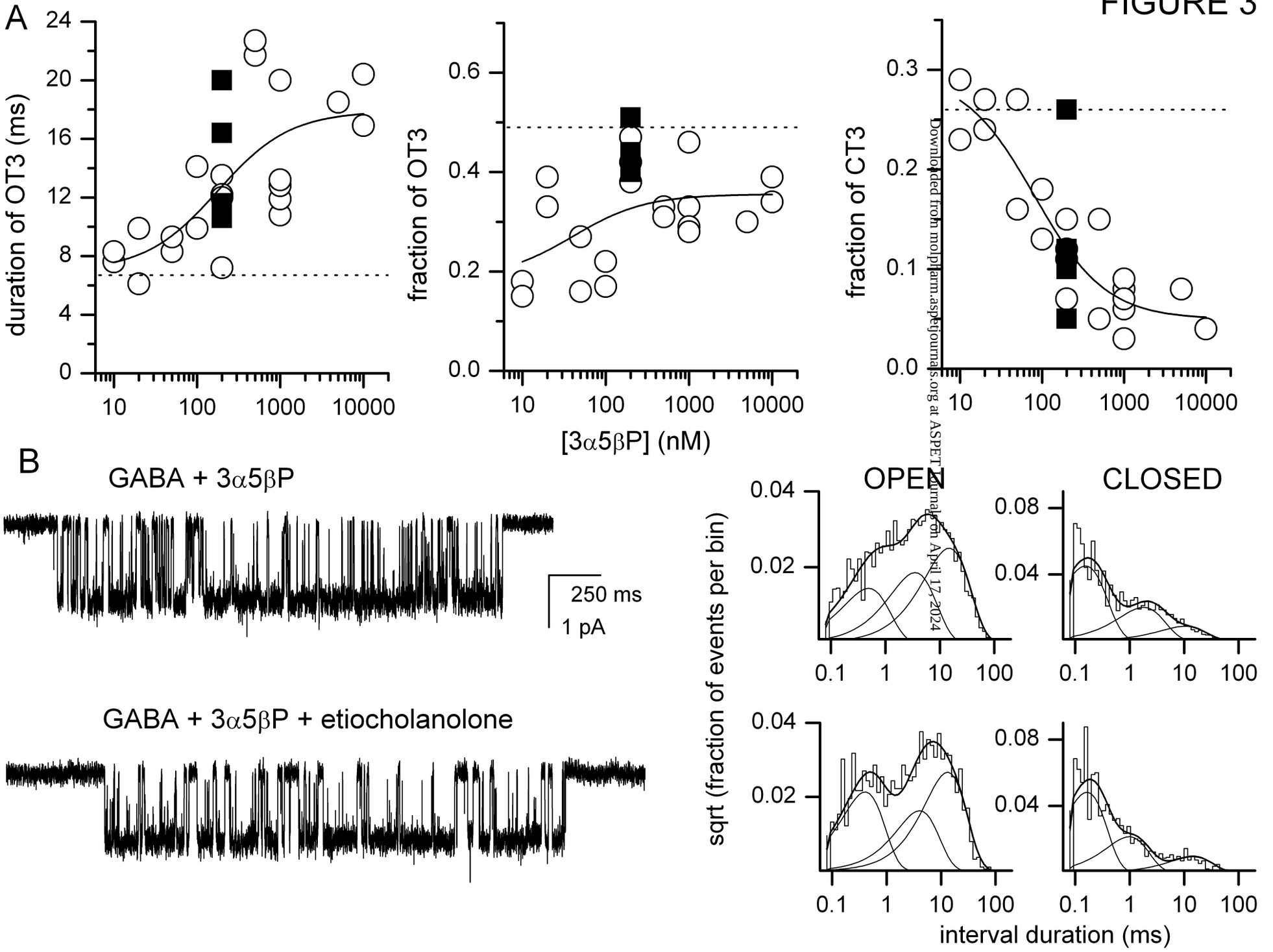

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FIGURE 4

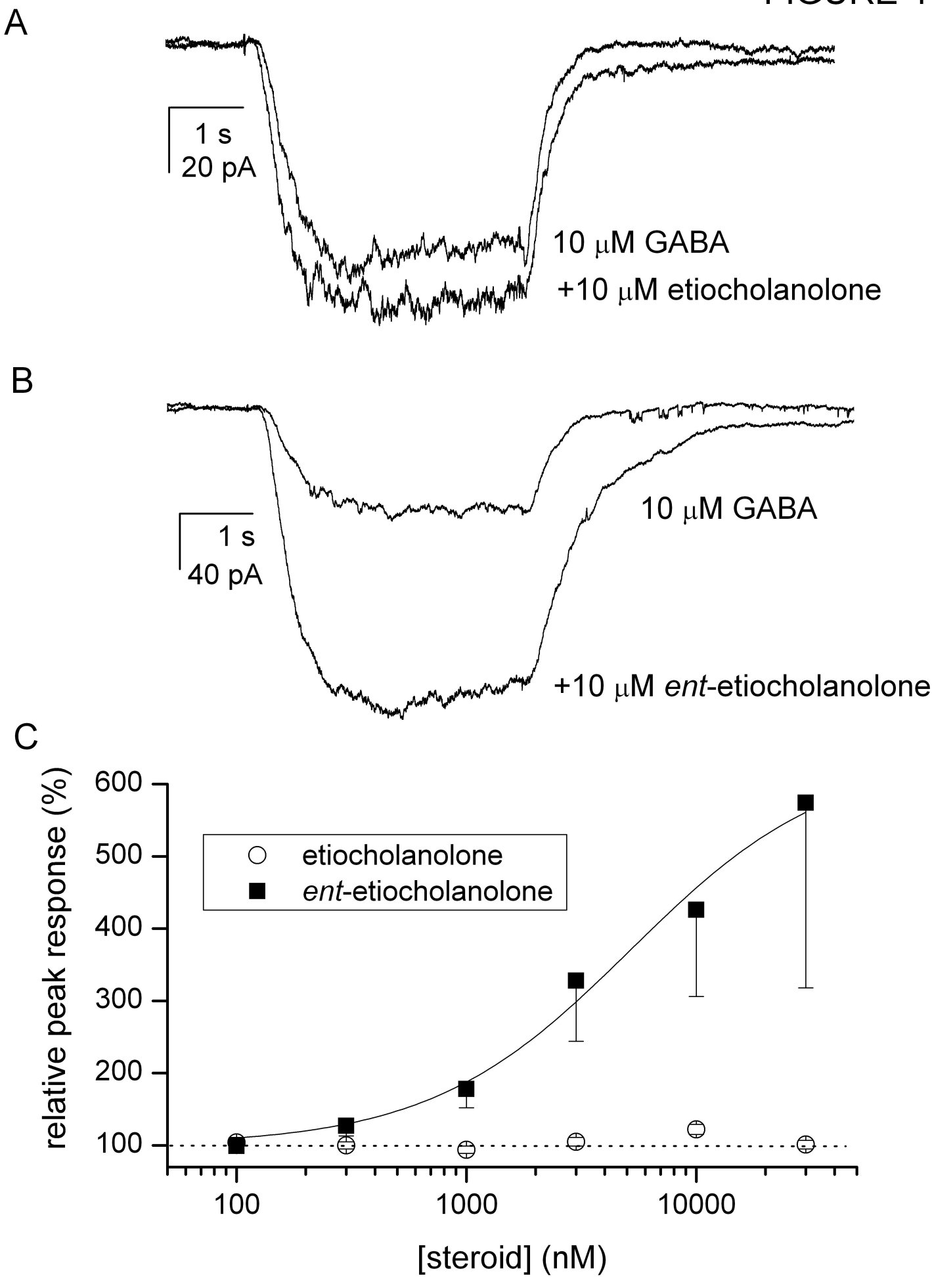

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FIGURE 5

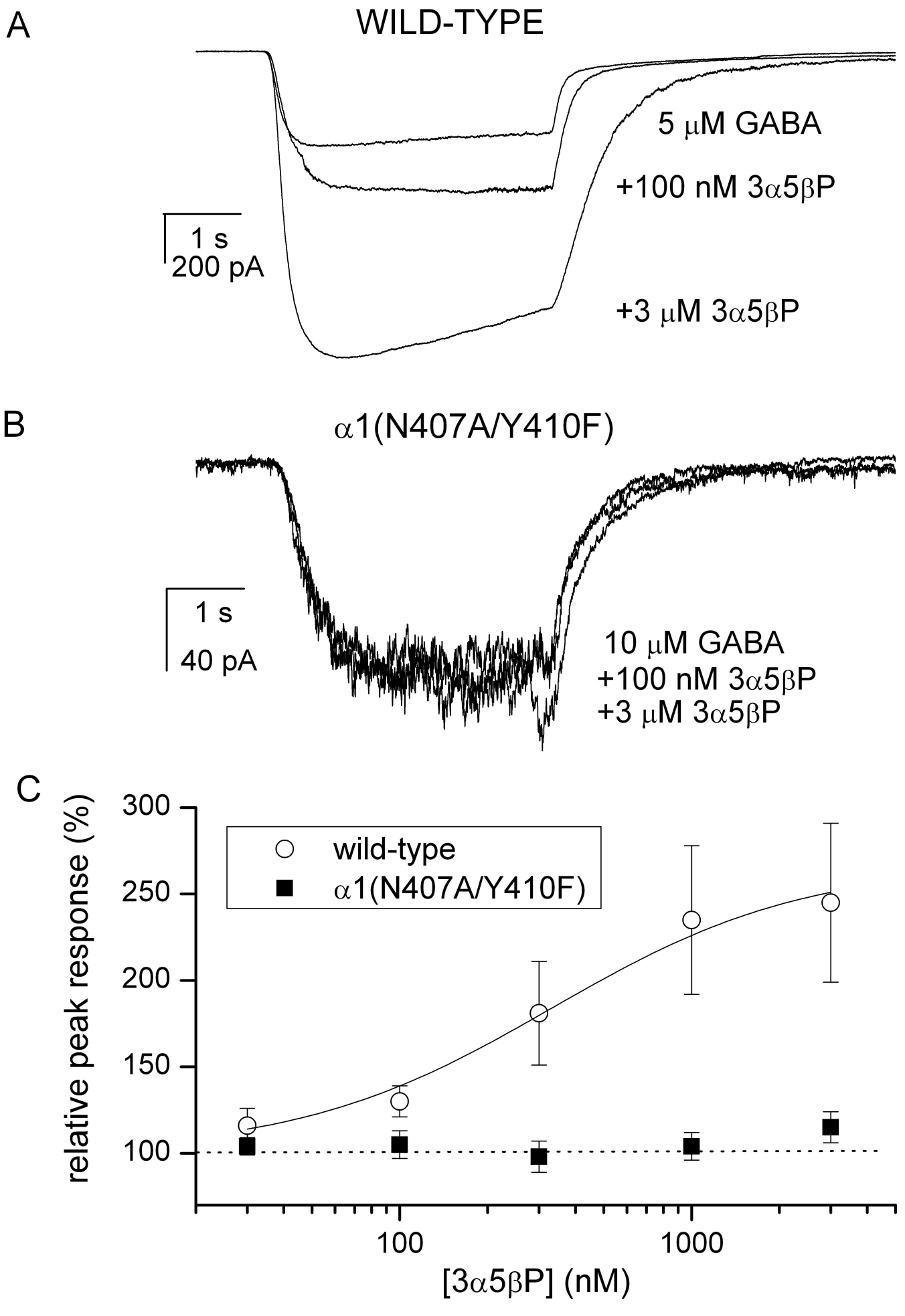

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FIGURE 6

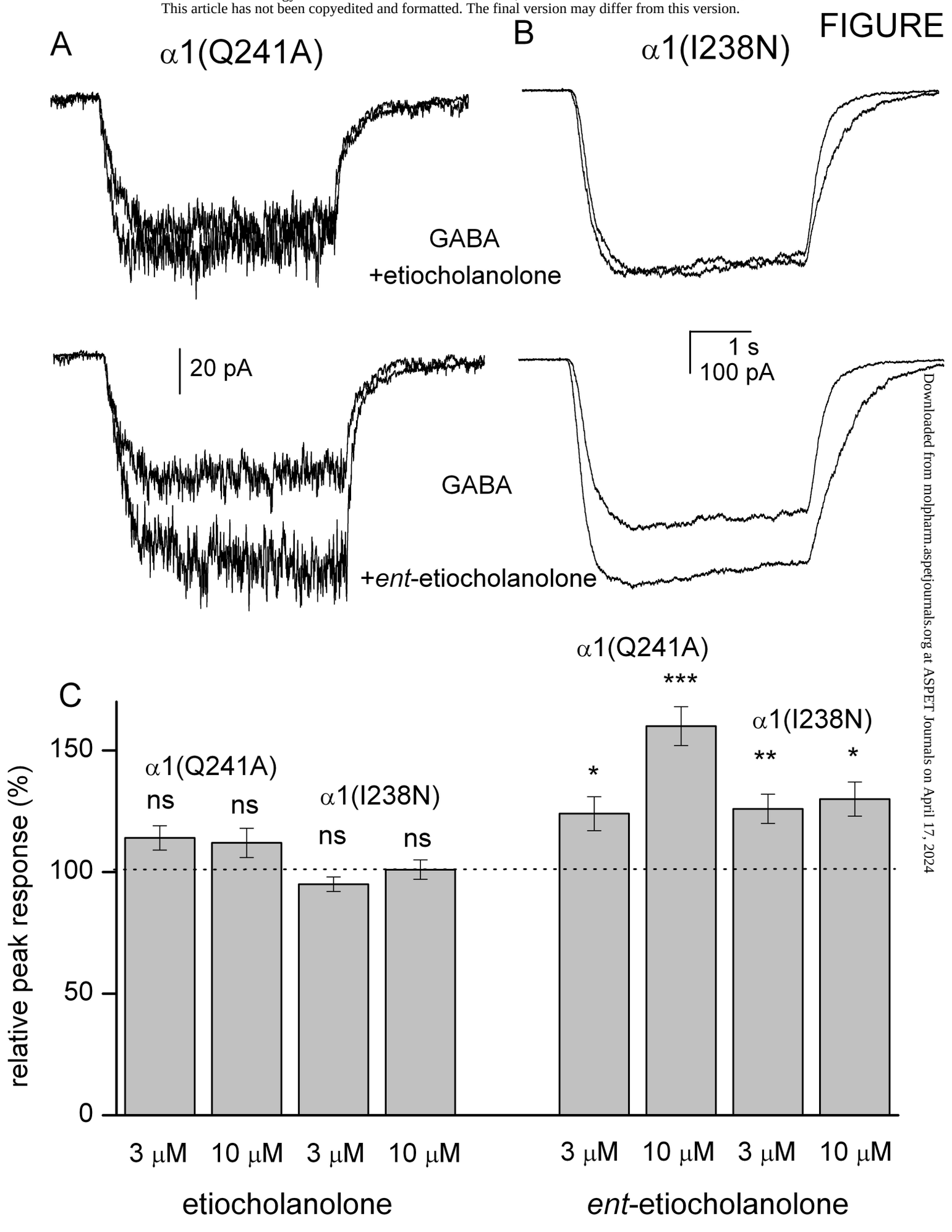

FIGURE 7