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Yusuke Sugasawa

Washington University School of Medicine in St. Louis

Wayland W.L. Cheng

Washington University School of Medicine in St. Louis

John R. Bracamontes

Washington University School of Medicine in St. Louis

Zi-Wei Chen

Washington University School of Medicine in St. Louis

Lei Wang

Washington University School of Medicine in St. Louis

See next page for additional authors

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Authors

Yusuke Sugasawa, Wayland W.L. Cheng, John R. Bracamontes, Zi-Wei Chen, Lei Wang, Allison L. Germann, Spencer R. Pierce, Thomas C. Senneff, Kathiresan Krishnan, David E. Reichert, Douglas F. Covey, Gustav Akk, and Alex S. Evers

Site-specific effects of neurosteroids on GABA_A receptor activation and desensitization

Yusuke Sugasawa¹, Wayland WL Cheng¹, John R Bracamontes¹, Zi-Wei Chen^{1,2}, Lei Wang¹, Allison L Germann¹, Spencer R Pierce¹, Thomas C Senneff¹, Kathiresan Krishnan³, David E Reichert^{2,4}, Douglas F Covey^{1,2,3,5}, Gustav Akk^{1,2}, Alex S Evers^{1,2,3*}

¹Department of Anesthesiology, Washington University in St. Louis, St. Louis, United States; ²Taylor Family Institute for Innovative Psychiatric Research, Washington University in St. Louis, St. Louis, United States; ³Department of Developmental Biology, Washington University in St. Louis, St. Louis, United States; ⁴Department of Radiology, Washington University in St. Louis, St. Louis, United States; ⁵Department of Psychiatry, Washington University in St. Louis, St. Louis, United States

Abstract This study examines how site-specific binding to three identified neurosteroid-binding sites in the $\alpha_1\beta_3$ GABA_A receptor (GABA_AR) contributes to neurosteroid allosteric modulation. We found that the potentiating neurosteroid, allopregnanolone, but not its inhibitory 3 β -epimer epi-allopregnanolone, binds to the canonical $\beta_3(+)-\alpha_1(-)$ intersubunit site that mediates receptor activation by neurosteroids. In contrast, both allopregnanolone and epi-allopregnanolone bind to intrasubunit sites in the β_3 subunit, promoting receptor desensitization and the α_1 subunit promoting effects that vary between neurosteroids. Two neurosteroid analogues with diazine moieties replacing the 3-hydroxyl (KK148 and KK150) bind to all three sites, but do not potentiate GABA_AR currents. KK148 is a desensitizing agent, whereas KK150 is devoid of allosteric activity. These compounds provide potential chemical scaffolds for neurosteroid antagonists. Collectively, these data show that differential occupancy and efficacy at three discrete neurosteroid-binding sites determine whether a neurosteroid has potentiating, inhibitory, or competitive antagonist activity on GABA_ARs.

*For correspondence: eversa@wustl.edu

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Introduction

Neurosteroids (NS) are endogenous modulators of brain development and function and are important mediators of mood (Belelli and Lambert, 2005; Mitchell et al., 2008; Represa and Ben-Ari, 2005; Grobin et al., 2006). Exogenously administered NS analogues have been clinically used as anesthetics and anti-depressants and have therapeutic potential as anti-epileptics, neuroprotective agents and cognitive enhancers (Belelli and Lambert, 2005; Mitchell et al., 2008; Akk et al., 2007; Reddy and Estes, 2016; Kharasch and Hollmann, 2015; Gunduz-Bruce et al., 2019; Zorumski et al., 2019). The principal target of NS is the γ -aminobutyric acid type A receptor (GABA_AR). NS can either activate or inhibit GABA_ARs. Positive allosteric modulatory NS (PAM-NS) such as allopregnanolone (3 α 5 α P) potentiate the effect of GABA on GABA_AR currents at low concentrations and directly activate the receptors at higher concentrations (Akk et al., 2007; Akk et al., 2010; Chen et al., 2019; Olsen, 2018). Negative allosteric modulatory NS (NAM-NS), such as epi-allopregnanolone (3 β 5 α P) or pregnenolone sulfate (PS) inhibit GABA_AR currents (Akk et al., 2001; Wang et al., 2002; Shen et al., 2000; Lundgren et al., 2003; Seljeset et al., 2018). In addition to

enhancing channel opening, PAM-NS increase the affinity of the GABA_AR for orthosteric ligand binding, an effect thought to be mechanistically linked to channel gating (Chen et al., 2019; Harrison et al., 1987a).

GABA_ARs are pentameric ligand-gated ion channels (pLGIC) composed of two α -subunits (α_{1-6}), two β -subunits (β_{1-3}) and one additional subunit (γ_{1-3} , δ , ϵ , θ or π) (Sigel and Steinmann, 2012; Sieghart, 2015; Olsen and Sieghart, 2008). Each subunit is composed of a large extracellular domain (ECD), a transmembrane domain (TMD) formed by four membrane-spanning helices (TM1-4), a long intracellular loop between TM3 and TM4, and a short extracellular C-terminus (Akk et al., 2007; Sigel and Steinmann, 2012; Sieghart, 2015; Laverty et al., 2019). NS modulate GABA_ARs by binding to sites within the TMDs (Belelli and Lambert, 2005; Mitchell et al., 2008; Akk et al., 2007; Reddy and Estes, 2016; Chen et al., 2019; Miller et al., 2017; Laverty et al., 2017; Hosie et al., 2006; Hosie et al., 2009; Chen et al., 2018; Sugasawa et al., 2019). Specifically, the α subunit TMDs are essential to the actions of PAM-NS (Chen et al., 2019; Miller et al., 2017; Laverty et al., 2017; Chen et al., 2018; Sugasawa et al., 2019). Mutagenesis studies in $\alpha_1\beta_2\gamma_2$ GABA_ARs have identified several residues in the α_1 subunit, notably Q242 and W246 in TM1, as critical to NS potentiation of GABA-elicited currents (Hosie et al., 2006; Hosie et al., 2009; Akk et al., 2008). Crystallographic studies have subsequently shown that, in homo-pentameric chimeric receptors in which the TMDs are derived from either α_1 (Laverty et al., 2017; Chen et al., 2018) or α_5 subunits (Miller et al., 2017), the NS 3 α ,21dihydroxy-5 α -pregnan-20-one (3 α 5 α -THDOC), pregnanolone and alphaxalone bind in a cleft between the α subunits, with the C3-hydroxyl substituent of the steroids interacting directly with Q242 in the α subunit (α Q242). PAM-NS activate these chimeric receptors, and their action is blocked by α Q242L and α Q242W mutations. These studies posit a single canonical intersubunit binding site for NS action that is conserved across the six α subunit isoforms (Miller et al., 2017; Laverty et al., 2017; Chen et al., 2018).

An alternative body of evidence suggests that PAM-NS modulation of GABA_AR function is mediated by multiple mechanisms and/or binding sites. Site-directed mutagenesis has identified multiple disparate residues on GABA_ARs that affect NS-induced activation, suggestive of two NS-binding sites: one site mediating potentiation of GABA responses and the other mediating direct activation (Hosie et al., 2006; Hosie et al., 2009). Single channel electrophysiological studies (Akk et al., 2007; Akk et al., 2010; Akk et al., 2004) as well as studies examining neurosteroid modulation of [³⁵S]t-butylbicyclophosphorothionate (TBPS) binding (Evers et al., 2010), have also identified multiple distinct effects of NS, with various structural analogues producing some or all of these effects, consistent with multiple NS-binding sites (Hosie et al., 2006; Hosie et al., 2009). Our recent photo-labeling studies have confirmed that there are multiple PAM-NS-binding sites on $\alpha_1\beta_3$ GABA_ARs (Chen et al., 2019). In addition to the canonical site at the interface between the TMDs of adjacent subunits (intersubunit site) (Chen et al., 2019; Miller et al., 2017; Laverty et al., 2017; Chen et al., 2018), we identified NS-binding sites within the α -helical bundles of both the α_1 and β_3 subunits (intrasubunit sites) of $\alpha_1\beta_3$ GABA_ARs (Chen et al., 2019). 3 α 5 α P binds to all three sites (Chen et al., 2019); mutagenesis of these sites suggests that the intersubunit and α_1 intrasubunit sites, but not the β_3 intrasubunit site, contribute to 3 α 5 α P PAM activity (Chen et al., 2019). A functional effect for NS binding to the β_3 intrasubunit site has not been identified.

The 3 α -hydroxyl (3 α -OH) group is critical to NS activation of GABA_ARs and 3 β -OH NS lack PAM activity (Akk et al., 2007; Wang et al., 2002). Indeed, many 3 β -OH NS are GABA_AR NAMs (Wang et al., 2002; Lundgren et al., 2003). While molecular docking studies have suggested that the 3 β -OH NS epi-pregnanolone (3 β 5 β P) should compete for binding with PAM-NS (Miller et al., 2017), 3 β -OH NS are non-competitive inhibitors with respect to GABA and 3 α -OH NS, indicating that they are unlikely to act at the canonical PAM-binding site (Akk et al., 2007; Wang et al., 2002). Steroids with a sulfate rather than a hydroxyl at the 3-carbon are also GABA_AR NAMs thought to act at sites distinct from GABA_AR PAMs (Akk et al., 2007; Akk et al., 2001; Wang et al., 2002; Seljeset et al., 2018; Park-Chung et al., 1999). The precise location of this site is unclear, but crystallographic studies have demonstrated a possible binding site between TM3 and TM4 on the intracellular end of the α -subunit TMD (Seljeset et al., 2018; Laverty et al., 2017). While 3 β -OH NS and PS both inhibit GABA_ARs, they likely act via interactions with distinct sites (Akk et al., 2007; Akk et al., 2001; Wang et al., 2002; Lundgren et al., 2003; Seljeset et al., 2018; Miller et al., 2017; Laverty et al., 2017).

The goal of the current study was to determine the specific sites underlying the PAM and NAM actions of NS. We hypothesized that various NS analogues preferentially bind to one or more of the three NS-binding sites in the $\alpha_1\beta_3$ GABA_AR, stabilizing distinct conformational states (i.e. resting, open or desensitized). To achieve this goal, we used two endogenous NS, the PAM-NS 3 α 5 α P and the NAM-NS 3 β 5 α P and two NS analogues, KK148 and KK150, in which a diazidine replaced the function-critical 3-OH group (Jiang et al., 2016). We examined site-specific NS binding and effects using NS photolabeling (Sugasawa et al., 2019; Budelier et al., 2017; Budelier et al., 2019; Cheng et al., 2018) and measurements of channel gating and orthosteric ligand binding. The NS lacking a 3 α -OH were devoid of PAM-NS activity, but surprisingly, KK148 and 3 β 5 α P enhanced the affinity of [³H]muscimol binding. We interpret this finding as evidence that these compounds preferentially bind to and stabilize desensitized receptors, since both open and desensitized GABA_AR exhibit enhanced orthosteric ligand-binding affinity (Chang et al., 2002).

The results show that 3 α 5 α P binds to the canonical $\beta(+)$ - $\alpha(-)$ intersubunit site, stabilizing the open state of the receptor, whereas the 3-diazirine NS (KK148 and KK150) bind to this site but do not promote channel opening, and 3 β 5 α P does not occupy this site. These data indicate that NS binding to the intersubunit sites is largely responsible for PAM activity and that the 3 α -OH is critical for NS activation. In contrast, 3 α 5 α P, 3 β 5 α P and the 3-diazirine NS all bind to both the α_1 and β_3 intrasubunit sites. Occupancy of the intrasubunit sites by 3 α 5 α P, 3 β 5 α P and KK148 promotes receptor desensitization. KK150 occupies all three NS-binding sites on $\alpha_1\beta_3$ GABA_AR, but produces minimal functional effect suggesting a possible scaffold for a general NS antagonist. These results shed new light on the mechanisms of NS allosteric modulation of channel function, and demonstrate a novel pharmacology in which related ligands bind to different subsets of functional sites on the same protein, each in a state-dependent manner, with the actions at these sites summing to produce a net physiological effect.

Results

Distinct patterns of NS potentiation and enhancement of muscimol binding

The endogenous NS, 3 α 5 α P is known to potentiate GABA-elicited currents (Figure 1A) and enhance [³H]muscimol binding to $\alpha_1\beta_3$ GABA_AR (Figure 1E; Chen et al., 2019; Harrison et al., 1987a). We examined a series of NS analogues with different stereochemistries or substituents in the 3- and 17-positions: 3 β 5 α P, KK148, and KK150 (structures shown in Figure 1B–D) for their ability to potentiate GABA-elicited currents and enhance orthosteric agonist ([³H]muscimol) binding. 3 β 5 α P is the 3 β -epimer of 3 α 5 α P. KK148 and KK150 are NS analogue photolabeling reagents, which have a 3-diazirine moiety instead of the 3-OH, and differ from each other by the stereochemistry of the 17-ether linkage (Jiang et al., 2016). We observed a discrepancy between the ability of these compounds to potentiate GABA-elicited currents and their ability to enhance [³H]muscimol binding in $\alpha_1\beta_3$ GABA_AR. None of the NS analogues lacking a 3 α -OH potentiated GABA-elicited currents (Figure 1B–D). However, both 3 β 5 α P and KK148 significantly enhanced [³H]muscimol binding (Figure 1E). KK150, in contrast, did not potentiate GABA-elicited currents and minimally enhanced [³H]muscimol binding (Figure 1D–E). Collectively, these data show that, NS analogues with different stereochemistry or substituents at the 3- and 17-positions show distinct patterns in modulation of $\alpha_1\beta_3$ GABA_AR currents and orthosteric ligand binding. We hypothesized that these patterns are a consequence of the various NS analogues stabilizing distinct conformational states of the GABA_AR, possibly by binding and acting at different sites. Notably, the compounds with a 3-OH (3 α 5 α P, 3 β 5 α P) are 10-fold more potent than those with a 3-diazirine (KK148, KK150) in enhancing [³H]muscimol binding (Figure 1E), suggesting that the 3-OH is an important determinant of binding affinity to the site(s) mediating these effects.

State-specific actions of NS analogues

To determine why 3 β 5 α P and KK148 enhance [³H]muscimol binding but do not potentiate $\alpha_1\beta_3$ GABA_AR currents, we first considered the possibility that 3 β 5 α P- and KK148-induced enhancement of [³H]muscimol binding is a selective effect on intracellular GABA_AR, since the radioligand binding assay was performed on total membrane homogenates, whereas the electrophysiological assays

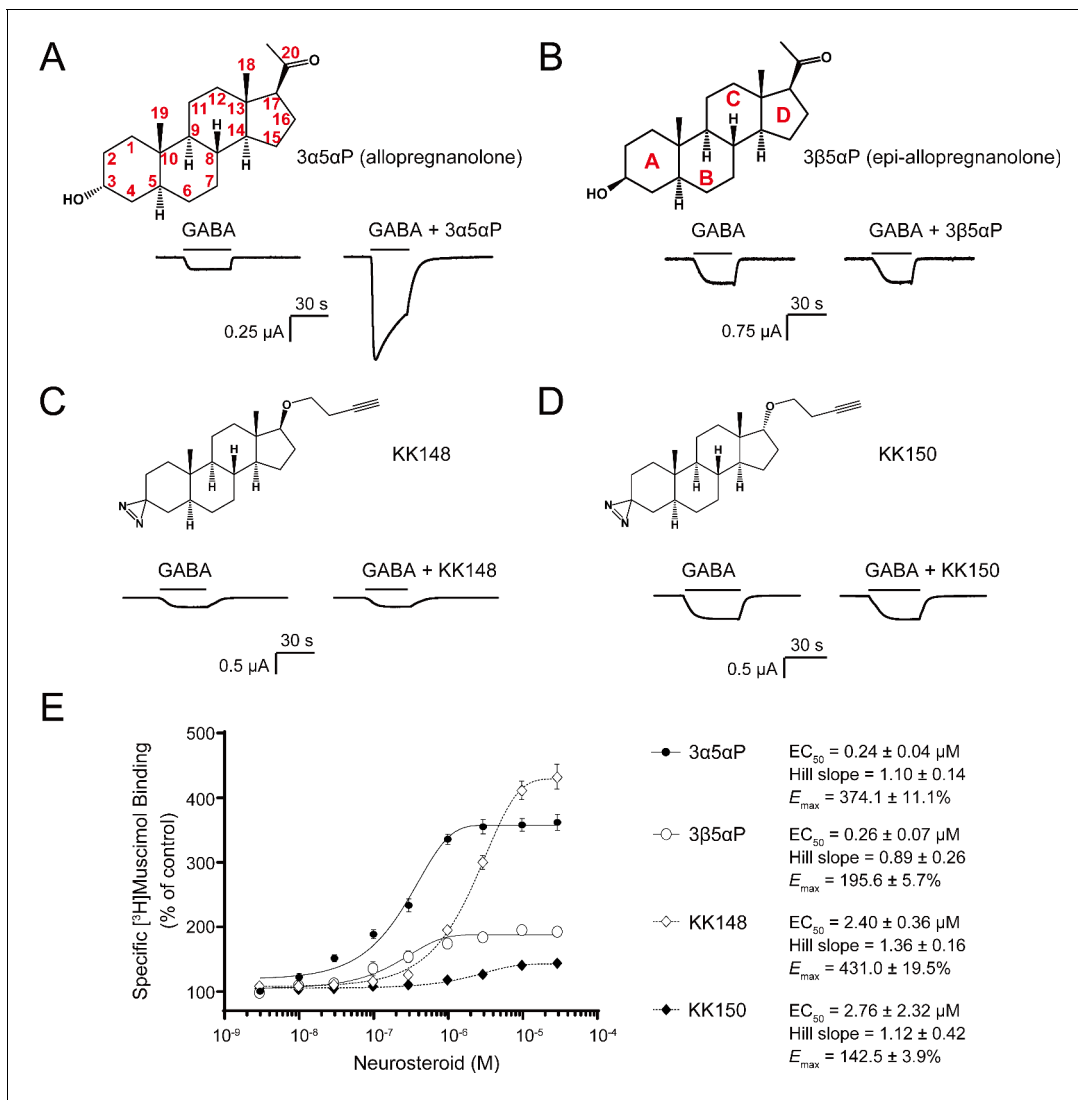


Figure 1. Distinct neurosteroid effects on potentiation of GABA_AR currents and modulation of $[^3\text{H}]$ muscimol binding. (A) Structure of allopregnanolone (3 α 5 α P) with carbon atoms numbered and sample current traces from $\alpha_1\beta_3$ GABA_AR activated by 0.3 μM GABA showing potentiation by 10 μM 3 α 5 α P. The traces were recorded from the same cell. (B), (C) and (D) Structures of epi-allopregnanolone (3 β 5 α P) with steroid rings labeled, neurosteroid analogue photolabeling reagents KK148 and KK150, respectively, and sample current traces from $\alpha_1\beta_3$ GABA_AR activated by 0.3 μM GABA showing the absence of potentiation by 10 μM neurosteroids. Each pair of traces was recorded from the same cell. (E) Concentration-response relationship for neurosteroid modulation of $[^3\text{H}]$ muscimol binding to $\alpha_1\beta_3$ GABA_AR. 3 nM–30 μM neurosteroids modulate $[^3\text{H}]$ muscimol (3 nM) binding in a concentration-dependent manner. Data points, EC_{50} , Hill slope and maximal effect value [E_{max} (% of control): 100% means no effect] are presented as mean \pm SEM ($n = 6$ for 3 α 5 α P and KK148; $n = 3$ for 3 β 5 α P and KK150).

The online version of this article includes the following figure supplement(s) for figure 1:

Figure supplement 1. Neurosteroid modulation of muscimol binding to intact cells.

report only from cell surface channels. NS are known to have effects on intracellular GABA_ARs and have been shown to accelerate GABA_AR trafficking (Abramian et al., 2014; Comenencia-Ortiz et al., 2014; Smith et al., 2007). To test this possibility, we examined $[^3\text{H}]$ muscimol binding in intact cells (i.e. binding to receptors only in the plasma membrane) (Vauquelin et al., 2015; Bylund et al., 2004; Bylund and Toews, 1993) compared to permeabilized cells (plasma membranes plus intracellular membranes). Notably, $[^3\text{H}]$ muscimol binding was twofold greater in permeabilized cells than in intact cells, indicating a significant population of intracellular GABA_ARs. KK148 enhanced $[^3\text{H}]$ muscimol binding in intact cells as much or more than in permeabilized cells, indicating

that this effect is not a result of selective NS actions on intracellular receptors (**Figure 1—figure supplement 1**).

A second possibility is that 3 β 5 α P and KK148 selectively bind to and stabilize a high-affinity non-conducting state, such as a pre-active (**Gielen and Corringer, 2018**) or a desensitized conformation of the GABA_AR. This is expected to result in inhibition of receptor function; however, the magnitude of the effect may be small under the experimental conditions used to generate the traces in **Figure 1**. To examine the inhibitory effect of these NS analogues, we activated $\alpha_1\beta_3$ GABA_AR with a saturating concentration (1 mM) of GABA and tested the effect of the NS on steady-state currents (**Germann et al., 2019a**). KK148 and 3 β 5 α P both decreased steady-state currents (**Figure 2A and C**), whereas KK150 did not (**Figure 2B**). To further delineate the electrophysiological effects of these compounds, we focused on 3 β 5 α P, since it is an endogenous NS and we had limited availability of KK148. Co-application of 3 β 5 α P with 1 mM GABA preferentially inhibited steady-state rather than peak currents (**Figure 2—figure supplement 1**). While this result is consistent with stabilization of a desensitized state rather than a pre-active state, it is ambiguous because it is possible that the steroid has a slower onset than GABA, thus minimizing the effect on peak current. Additional evidence that 3 β -NAM-NS stabilize a desensitized state includes studies examining their effects on inhibitory post-synaptic currents (**Wang et al., 2002**) and single channel currents (**Akk et al., 2001**). The evidence that NAM-NS stabilize a desensitized rather than a pre-active state is more thoroughly explored in the Discussion. In the ensuing text, we refer to the inhibition of steady-state current as desensitization.

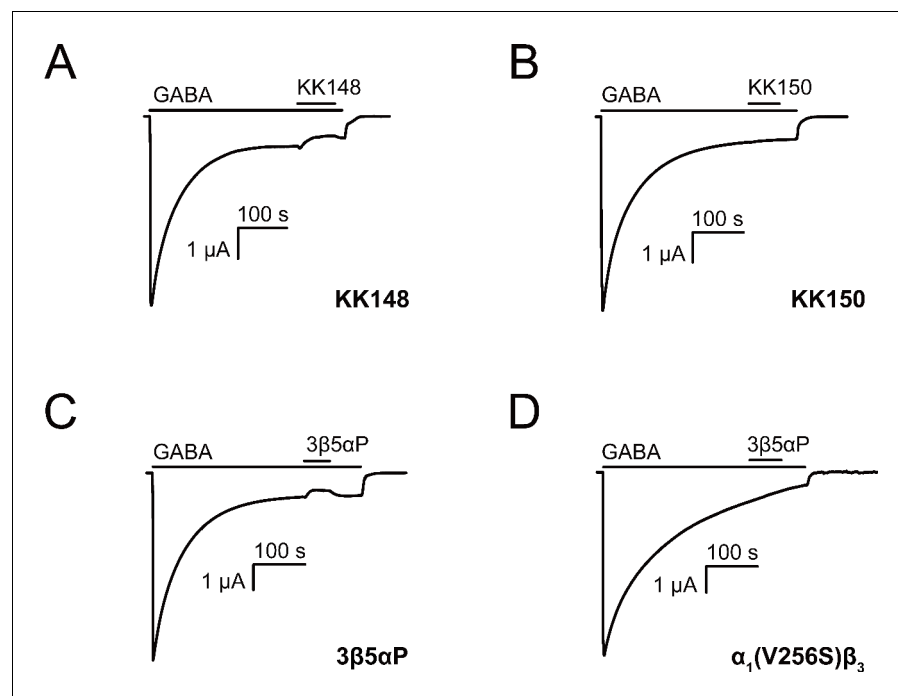


Figure 2. Neurosteroids promote steady-state desensitization of $\alpha_1\beta_3$ GABA_ARs. Representative traces showing the effects of KK148, KK150 and epi-allopregnanolone (3 β 5 α P) on maximal steady-state GABA-elicited currents. $\alpha_1\beta_3$ GABA_ARs expressed in *Xenopus laevis* oocytes were activated with 1 mM GABA to maximally activate GABA_AR current. (A–C) The effect of KK148 (10 μ M), KK150 (10 μ M) and 3 β 5 α P (3 μ M) on steady-state current. (D) The effect of 3 β 5 α P (3 μ M) on steady-state current in $\alpha_1\beta_3$ GABA_ARs containing the α_1 V256S mutation, known to eliminate NS-induced desensitization. The results show that 3 β 5 α P and KK148 reduce steady-state currents, consistent with enhanced desensitization, whereas KK150 does not. The effect of 3 β 5 α P on steady-state currents is eliminated by the α_1 V256S mutation, consistent with 3 β 5 α P enhancing desensitization rather than producing channel block.

The online version of this article includes the following figure supplement(s) for figure 2:

Figure supplement 1. Co-application of epi-allopregnanolone with a saturating concentration of GABA.

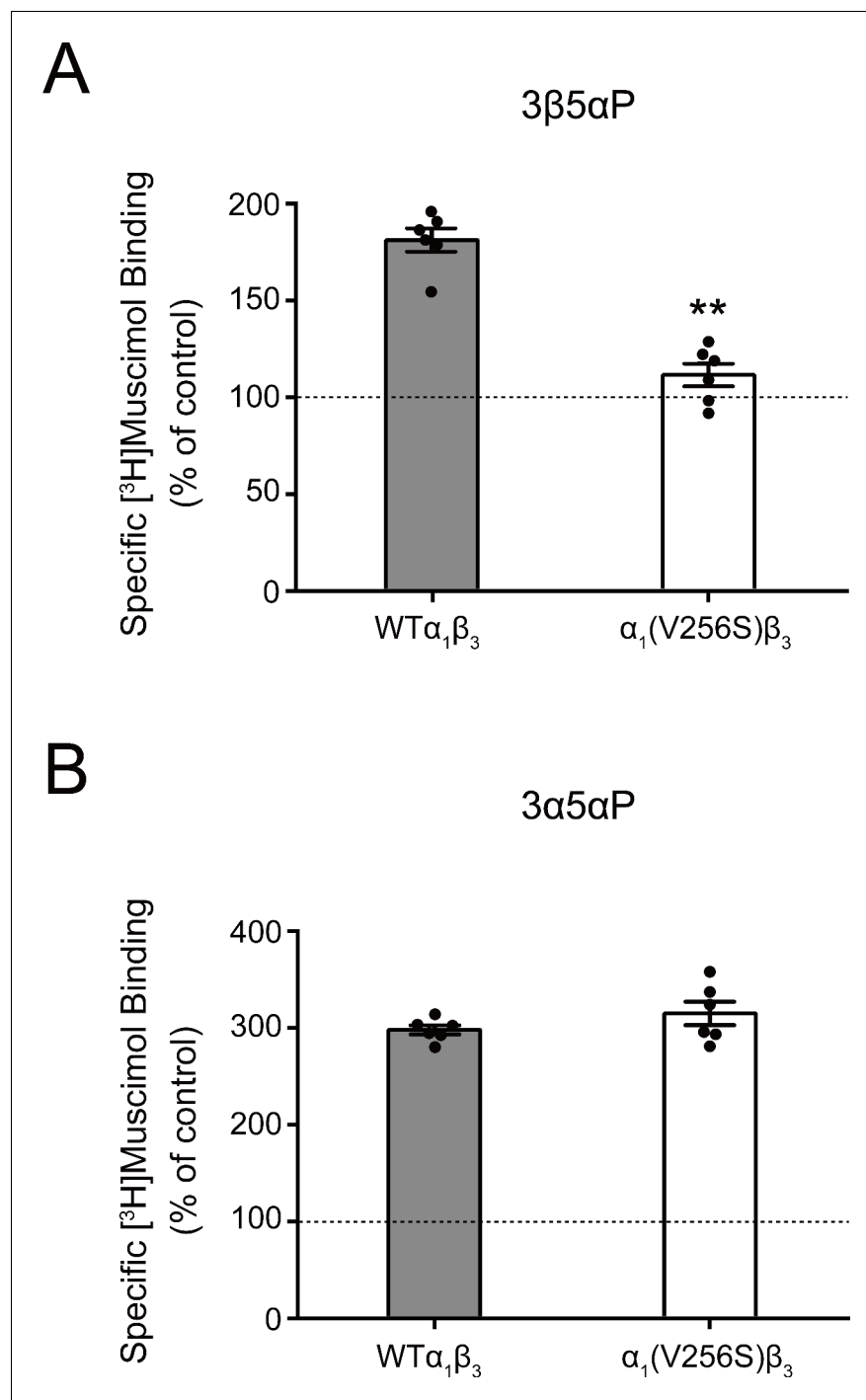


Figure 3. Effect of $\alpha_1(V256S)\beta_3$ mutation on neurosteroid enhancement of [³H]muscimol binding. (A) Enhancement of specific [³H]muscimol (3 nM) binding to $\alpha_1\beta_3$ GABA_AR WT by 10 μ M epi-allopregnanolone (3β5αP) is absent in $\alpha_1(V256S)\beta_3$ GABA_AR. (B) Enhancement of [³H]muscimol binding by 10 μ M allopregnanolone (3α5αP) is unaffected by the α_1V256S mutation. These data indicate that 3β5αP enhancement of orthosteric ligand binding requires receptor desensitization, whereas 3α5αP does not. Statistical differences are compared using unpaired t-test ($n = 6, \pm$ SEM). ** $p < 0.01$ vs. WT.

The inhibitory effect of 3β5αP was not observed in receptors with the $\alpha_1(V256S)$ TM2 pore-lining mutation, which was previously shown to remove the inhibitory effects of sulfated steroids (Akk et al., 2001; Wang et al., 2002; Figure 2D). Although both 3α5αP and 3β5αP enhance [³H]

muscimol binding, the former predominantly results in receptor activation, whereas the latter results in inhibition. Consistent with this, the $\alpha_1(V256S)\beta_3$ mutation which abolishes NS-induced inhibition (Akk et al., 2001; Wang et al., 2002) eliminated [3H]muscimol binding enhancement by $3\beta 5\alpha P$ but not $3\alpha 5\alpha P$ (Figure 3). We infer that $3\alpha 5\alpha P$ increases [3H]muscimol binding by stabilizing an active state of the receptor. In contrast, $3\beta 5\alpha P$ increases [3H]muscimol binding by stabilizing a desensitized state of the receptor; this effect is eliminated in the $\alpha_1(V256S)\beta_3$ receptor. The mechanisms of enhancement of [3H]muscimol binding by allosteric activators and inhibitors are described in detail in our recent publication (Akk et al., 2020). Collectively, these data indicate that $3\beta 5\alpha P$ and KK148 enhance orthosteric ligand affinity by stabilizing a desensitized state of the GABA_AR.

Quantitative comparison of the effects of $3\beta 5\alpha P$ on [3H]muscimol binding and receptor desensitization

While there is qualitative agreement between the relative effects of the various NS analogues on orthosteric ligand binding and receptor desensitization, there is a quantitative discrepancy in the magnitude of the effects. For example, $3\beta 5\alpha P$ enhances [3H]muscimol binding by two-fold (Figure 1E), whereas it reduces steady-state current by only ~25% (Figure 2C). To address this difference, we considered that the radioligand binding and electrophysiological assays are performed under different experimental conditions. The radioligand-binding studies are performed using low [3H]muscimol concentrations to allow for sufficient dynamic range of ligand binding. In contrast, the desensitization experiments are performed at high orthosteric ligand (GABA) concentration to achieve high peak open probability and steady-state receptor desensitization, thus minimizing the number of channels in the resting state. To address the quantitative differences in results from the two assays, we analyzed the electrophysiological data in the framework of the three-state Resting-Open-Desensitized model (Germann et al., 2019a; Germann et al., 2019b). We assumed that both the open and desensitized states had higher affinity for muscimol than the resting state, and that the affinities were similar and could be treated as equal. We then calculated the predicted occupancy of the high-affinity states ($P_{open} + P_{desensitized}$) using parameters derived from the functional responses, to compare to the observed changes in binding. The raw current amplitudes of peak and steady-state responses were converted to units of open probability as described previously in detail (Eaton et al., 2016), and the probabilities of being in the open (P_{open}) or desensitized ($P_{desensitized}$) states were calculated for different experimental conditions (see Materials and methods).

Application of 1 mM GABA elicited a current response that had a peak P_{open} of 0.71 ± 0.25 (mean \pm SD; $n = 16$). The P_{open} of the steady-state response was 0.121 ± 0.033 ($n = 7$), that was reduced to 0.077 ± 0.013 ($n = 5$) with 3 μM $3\beta 5\alpha P$. Analysis of steady-state currents using the Resting-Open-Desensitized model indicates that the steady-state $P_{desensitized}$ is 0.829 in the presence of GABA, and 0.892 in the presence of GABA + steroid. The relatively small increase in the sum of ($P_{open} + P_{desensitized}$) (from 0.95 to 0.97) is due to the use of saturating GABA in these experiments.

To compare the data from the radioligand binding and electrophysiology experiments, we exposed oocytes containing $\alpha_1\beta_3$ GABA_ARs to 20 nM muscimol and recorded currents before and after co-application of 3 μM $3\beta 5\alpha P$. The percent reduction in steady-state current following $3\beta 5\alpha P$ exposure was measured and used to estimate the relative probabilities of resting, open and desensitized receptors. The application of 20 nM muscimol elicited a peak response with P_{open} of 0.012 ± 0.004 ($n = 6$). The steady-state P_{open} was 0.011 ± 0.004 . In the same cells, subsequent exposure to 3 μM $3\beta 5\alpha P$ reduced the steady-state P_{open} to 0.009 ± 0.004 ($p = 0.0174$; paired t-test). The calculated steady-state $P_{desensitized}$ was 0.1001 in the presence of muscimol, and 0.2168 in the presence of muscimol + $3\beta 5\alpha P$. Thus, there is a predicted two-fold increase in the sum of ($P_{open} + P_{desensitized}$) when the steroid is combined with muscimol, consistent with the doubling of muscimol binding caused by $3\beta 5\alpha P$ in the [3H]muscimol binding experiments (Figure 1E). While the measured changes in current are small, they are precise because each experiment served as its own control; a steady-state current was achieved during continuous agonist administration and the response to $3\beta 5\alpha P$ was then measured. Overall, these data indicate that when $P_{resting}$ is high (low orthosteric ligand concentration), an agent that stabilizes desensitized receptors may produce a small decrease in steady-state current, but a relatively large increase in the occupancy of desensitized state, at the expense of resting receptors. Conversely, with high orthosteric ligand concentrations (low $P_{resting}$), a desensitizing ligand produces a relatively larger change in steady-state current as open receptors

are converted to desensitized receptors with minimal effect on the sum occupancy of high-affinity states.

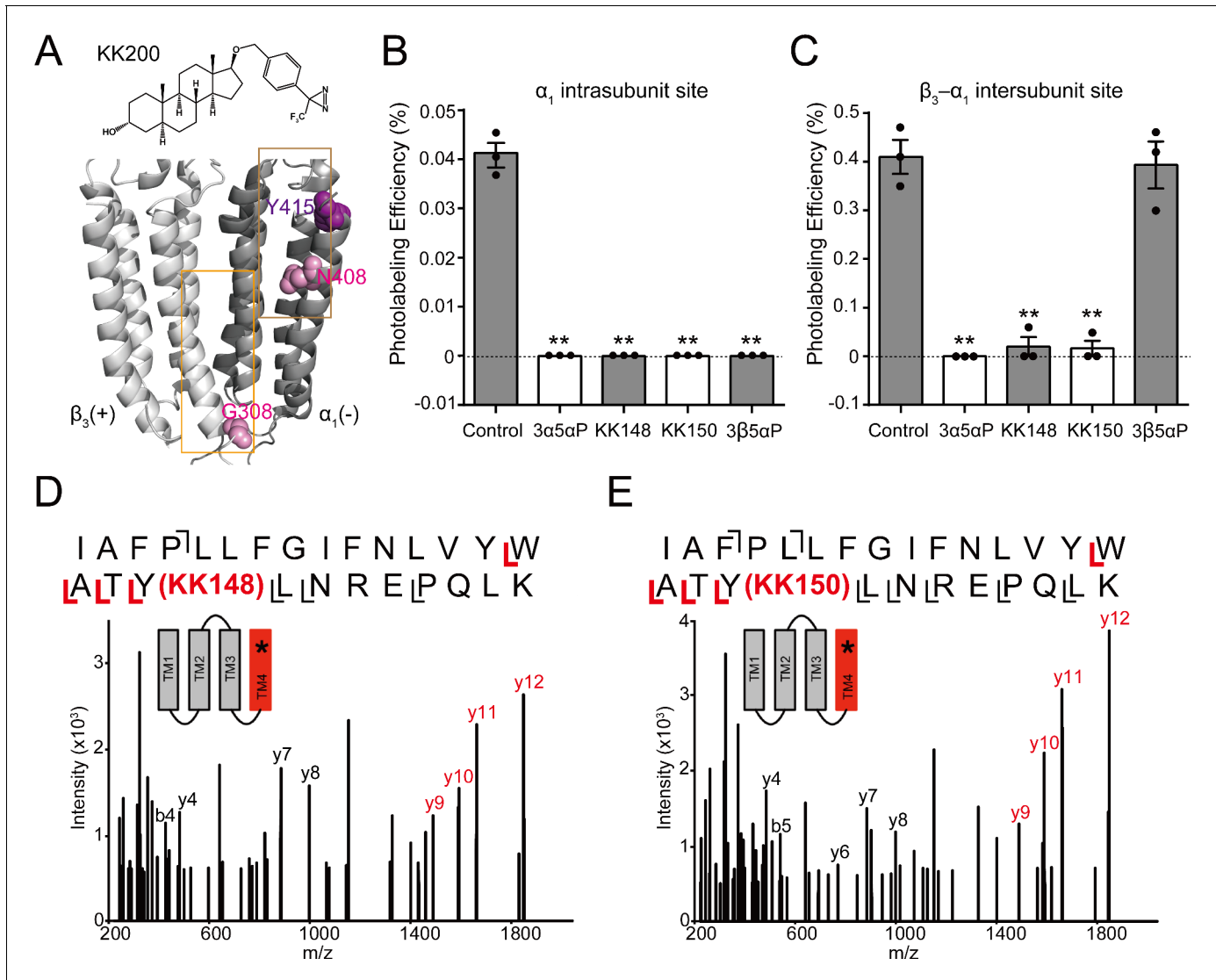


Figure 4. Competitive prevention of neurosteroid photolabeling at an intersubunit and intrasubunit site. (A) Structures of the neurosteroid photolabeling reagent KK200 and the $\alpha_1\beta_3$ GABA_AR-TMDs highlighting the residues G308 in the $\beta_3(+)$ - $\alpha_1(-)$ intersubunit site and N408 in the α_1 intrasubunit site previously identified by KK200 photolabeling in pink. Shown in purple is Y415 in the α_1 intrasubunit site, which is photolabeled by KK148 and KK150. Adjacent $\beta_3(+)$ and $\alpha_1(-)$ subunits are shown and the channel pore is behind the subunits. (B) Photolabeling efficiency of α_1 subunit TM4 (α_1 intrasubunit site) in $\alpha_1\beta_3$ GABA_AR by 3 μ M KK200 in the absence or presence of 30 μ M allopregnanolone (3 α 5 α P), KK148, KK150, and epi-allopregnanolone (3 β 5 α P). Statistical differences are analyzed using one-way ANOVA with Bonferroni's multiple comparisons test ($n = 3, \pm$ SEM). ** $p < 0.01$ vs. control. (C) Same as (B) for β_3 subunit TM3 [$\beta_3(+)$ - $\alpha_1(-)$ intersubunit site, $n = 3, \pm$ SEM]. (D) HCD fragmentation spectrum of the α_1 subunit TM4 tryptic peptide photolabeled by 30 μ M KK148. Red and black indicate fragment ions that do or do not contain KK148, respectively. The schematic highlight in red identifies the TMD being analyzed and the asterisk denotes the approximate location of KK148. (E) Same as (D) photolabeled by 30 μ M KK150.

The online version of this article includes the following figure supplement(s) for figure 4:

Figure supplement 1. Extracted ion chromatograms of labeled and unlabeled β_3 subunit TM4 peptides.

Figure supplement 2. Fragmentation spectrum of unlabeled α_1 subunit TM4 peptide.

Binding site selectivity for NS analogues

To determine whether KK148 and $3\beta 5\alpha P$ stabilize a desensitized conformation of the GABA_AR by selectively binding to one or more of the identified NS-binding sites on the GABA_AR (Chen et al., 2019), we first determined which of the identified NS sites they bind. We have previously shown that the $3\alpha 5\alpha P$ -analogue photolabeling reagent, KK200 labels the $\beta_3(+)-\alpha_1(-)$ intersubunit (β_3G308) and α_1 intrasubunit (α_1N408) sites on $\alpha_1\beta_3$ GABA_ARs (Figure 4A), and that photolabeling can be prevented by a 10-fold excess of $3\alpha 5\alpha P$ (Chen et al., 2019). As a first step to determine the binding sites for $3\beta 5\alpha P$, KK148 or KK150, we examined whether a 10-fold excess of these compounds (30 μM) prevented KK200 (3 μM) photolabeling of either binding site. Photolabeling was performed on membranes from HEK293 cells transfected with epitope-tagged $\alpha_{1His-FLAG}\beta_3$ receptors, mimicking

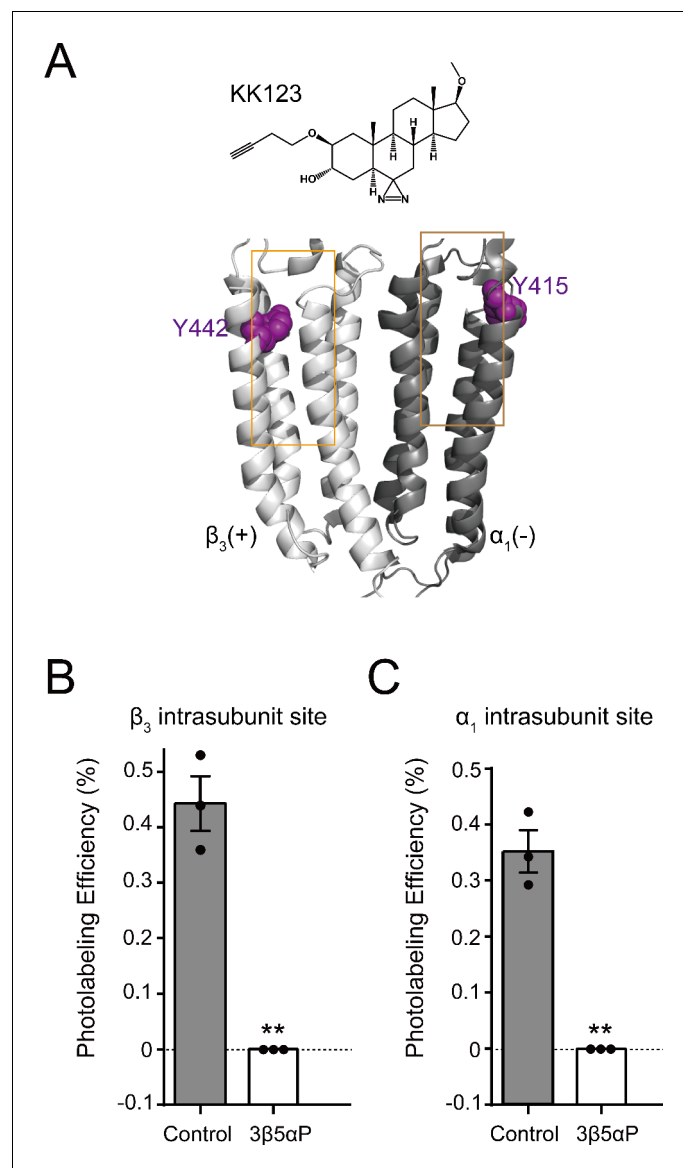


Figure 5. Epi-allopregnanolone prevents neurosteroid photolabeling at the α_1 and β_3 intrasubunit sites. (A) Structures of the neurosteroid photolabeling reagent KK123 and the $\alpha_1\beta_3$ GABA_AR-TMDs highlighting the residues Y442 in the β_3 intrasubunit site and Y415 in the α_1 intrasubunit site previously identified by KK123 photolabeling in purple. Adjacent $\beta_3(+)$ and $\alpha_1(-)$ subunits are shown and the channel pore is behind the subunits. (B) Photolabeling efficiency of β_3 subunit TM4 (β_3 intrasubunit site) in $\alpha_1\beta_3$ GABA_AR by 3 μM KK123 in the absence or presence of 30 μM epi-allopregnanolone (3 $\beta 5\alpha P$). Statistical differences are compared using unpaired *t*-test (*n* = 3, \pm SEM). ***p* < 0.01 vs. control. (C) Same as (B) for α_1 subunit TM4 (α_1 intrasubunit site, *n* = 3, \pm SEM).

the conditions used in the [^3H]muscimol binding assays and photolabeled residues were identified and labeling efficiency was determined using middle-down mass spectrometry (Chen et al., 2019). KK148, KK150, $3\alpha 5\alpha\text{P}$ and $3\beta 5\alpha\text{P}$ all prevented KK200 photolabeling of $\alpha_1\text{N408}$ in the α_1 intrasubunit site (Figure 4B), consistent with their binding to this site. In contrast, KK148, KK150 and $3\alpha 5\alpha\text{P}$ but not $3\beta 5\alpha\text{P}$ prevented labeling of $\beta_3\text{G308}$ in the intersubunit site (Figure 4C), indicating that $3\beta 5\alpha\text{P}$ does not bind to the intersubunit site. Similarly, $3\beta 5\alpha\text{P}$ did not prevent labeling of the intersubunit site by a similar NS-analogue photolabeling reagent in detergent-solubilized GABA $_A$ Rs (Jayakar et al., 2020).

The KK148- and KK150-photolabeled (30 μM) samples were also analyzed to directly identify the sites of adduction. In both the KK148- and KK150-labeled samples, photolabeled peptides were identified from the TM4 helices of both the α_1 and β_3 subunits. The labeled peptides had longer chromatographic elution times than the corresponding unlabeled peptides and corresponded with high mass accuracy (<20 ppm) to the predicted mass of the unlabeled peptides plus the add weight minus N_2 of KK148 or KK150 (Figure 4—figure supplement 1). Product ion (MS2) spectra of the KK148- and KK150-labeled peptides from the α_1 subunit identified the labeled residue as Y415 for both KK148 and KK150 with photolabeling efficiencies of 0.77% and 0.62%, respectively (Figure 4D–E, Figure 4—figure supplement 2); Y415 is the same residue labeled by KK123 at the α_1 intrasubunit site (Chen et al., 2019). The KK148 and KK150 labeled peptides in TM4 of the β_3 subunit and corresponding unlabeled peptide were identified by fragmentation spectra as $\beta_3\text{TM4 1426-N445}$. These data support labeling of the β_3 intrasubunit site by KK148 and KK150. Fragmentation spectra of the peptide-sterol adducts were not adequate to determine the precise labeled residue because of low photolabeling efficiency (0.13% for KK148; 0.19% for KK150, Figure 4—figure supplement 1). No photolabeled peptides were identified in the $\beta_3(+)-\alpha_1(-)$ intersubunit site. This is likely because KK148 and KK150, similar to KK123, utilize an aliphatic diazine that preferentially labels nucleophilic residues (Sugasawa et al., 2019; Budelier et al., 2017; Das, 2011); such residues are not present in the intersubunit site.

We have also shown that KK123 labeling of the α_1 intrasubunit ($\alpha_1\text{Y415}$) and β_3 intrasubunit ($\beta_3\text{Y442}$) sites (Figure 5A) can be prevented by a 10fold excess of $3\alpha 5\alpha\text{P}$ (Chen et al., 2019). We thus examined whether $3\beta 5\alpha\text{P}$ (30 μM) inhibited photolabeling by KK123 (3 μM). $3\beta 5\alpha\text{P}$ completely inhibited KK123 photolabeling at both intrasubunit sites (Figure 5B–C). Collectively, the data show that KK148, KK150 and $3\alpha 5\alpha\text{P}$ bind to all three of the identified NS-binding sites. In contrast, $3\beta 5\alpha\text{P}$ selectively binds to the two intrasubunit binding sites, but not to the canonical $\beta_3(+)-\alpha_1(-)$ intersubunit site.

Orthosteric ligand binding enhancement by NS analogues is mediated by distinct sites

To determine which of the previously identified binding sites contributes to NS enhancement of [^3H]muscimol binding, we performed site-directed mutagenesis of the NS-binding sites previously determined by photolabeling (Figure 6A; Chen et al., 2019). Specifically, $\alpha_1(\text{Q242L})\beta_3$ targets the $\beta_3(+)-\alpha_1(-)$ intersubunit site, $\alpha_1(\text{N408A/Y411F})\beta_3$ and $\alpha_1(\text{V227W})\beta_3$ the α_1 intrasubunit site, and $\alpha_1\beta_3(\text{Y284F})$ the β_3 intrasubunit site. None of these mutations produced a significant change in [^3H]muscimol K_d (Figure 7B and Figure 7—source data 1). Accordingly, concentration-dependent NS effects were assayed at a fixed concentration of [^3H]muscimol (3 nM; $\sim\text{EC}_5$). It should be noted that earlier studies showed two-component binding curves for [^3H]muscimol in brain membranes, with NS causing an increase in the B_{max} of the high-affinity component (Harrison and Simmonds, 1984). In contrast, our results with expressed $\alpha_1\beta_3$ GABA $_A$ Rs show a single-component [^3H]muscimol binding curve with NS producing an increase in muscimol affinity. Our results are similar to results reported with expressed $\alpha_1\beta_3\gamma_2$ GABA $_A$ Rs, where allosteric modulators increased the affinity of a single-component [^3H]muscimol binding curve (Dostalova et al., 2014). Whether the complex [^3H]muscimol binding curves observed in brain is the result of heterogeneity of receptor subtypes or multiple states of the GABA $_A$ R is unresolved.

Mutations in the $\beta_3(+)-\alpha_1(-)$ intersubunit and α_1 intrasubunit sites decreased $3\alpha 5\alpha\text{P}$ enhancement of [^3H]muscimol binding by $\sim 80\%$, while mutation of the β_3 intrasubunit site led to a small decrease (Figure 6B, Table 1). The residual enhancement of [^3H]muscimol binding observed in receptors with mutations in the intersubunit or α_1 intrasubunit site occurs at 10fold higher concentrations of $3\alpha 5\alpha\text{P}$ than wild-type (WT) and receptors with mutations in the β_3 intrasubunit site (Table 1), suggesting

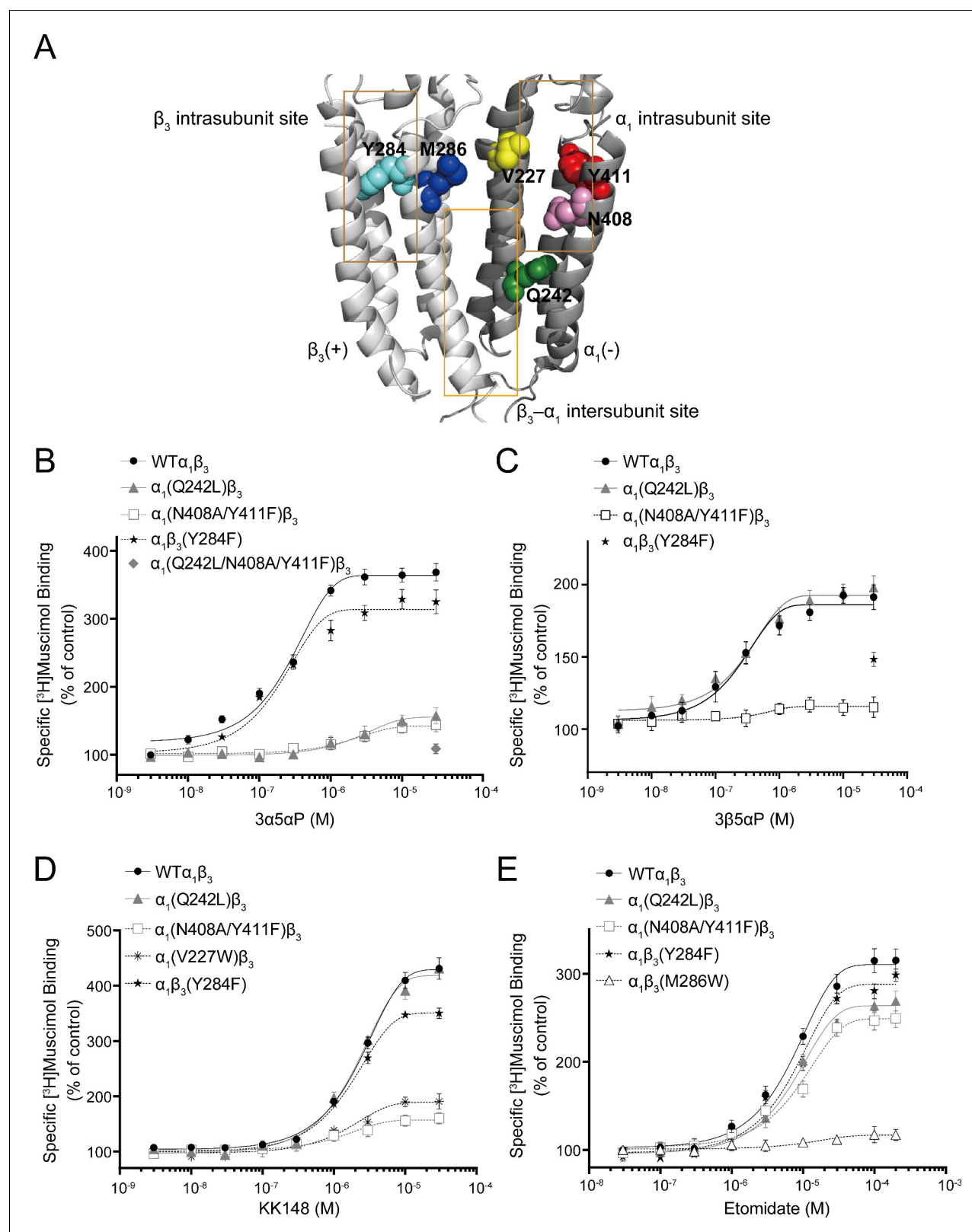


Figure 6. Effect of mutations in $\alpha_1\beta_3$ GABA $_A$ R on neurosteroid modulation of [3 H]muscimol binding. (A) Structure of the $\alpha_1\beta_3$ GABA $_A$ R-TMD highlighting the residues where mutations were made in putative binding sites for neurosteroids (Q242-green for β_3 - α_1 intersubunit site; V227-yellow, N408-pink and Y411-red for α_1 intrasubunit site; Y284-cyan for β_3 intrasubunit site) and M286-blue for etomidate. Adjacent $\beta_3(+)$ and $\alpha_1(-)$ subunits are shown and the channel pore is behind the subunits. (B) Concentration-response relationship for the effect of 3 nM–30 μ M allopregnanolone (3 α 5 α P) on

Figure 6 continued on next page

Figure 6 continued

[³H]muscimol (3 nM) binding to $\alpha_1\beta_3$ GABA_AR WT and indicated mutants. Data points represent mean \pm SEM ($n = 6$). (C), (D) and (E) Same as (B) for 3 nM–30 μ M epi-allopregnanolone (3 β 5 α P) ($n = 3$), KK148 ($n = 6$) and 30 nM–200 μ M etomidate ($n = 6$), respectively. The data for WT in panels 6B and 6D is a replot of the same data shown in **Figure 1E**.

The online version of this article includes the following figure supplement(s) for figure 6:

Figure supplement 1. Time course of neurosteroid modulation of muscimol binding.

that 3 α 5 α P binds to the β_3 intrasubunit site with lower affinity. In contrast, mutations in the α_1 and β_3 intrasubunit sites, but not the intersubunit site decreased the enhancement of [³H]muscimol binding by 3 β 5 α P and KK148 (**Figure 6C–D, Table 1**). To confirm that the effect of these mutations on NS effect are steroid-specific, we also tested their effect on etomidate, which enhances [³H]muscimol binding in $\alpha_1\beta_3$ GABA_ARs and acts through a binding site distinct from NS (Li et al., 2006; Jayakar et al., 2019). The mutations targeting NS-binding sites resulted in modest decreases in [³H]muscimol binding enhancement by etomidate; however, the $\alpha_1\beta_3$ (M286W) mutation which abolishes etomidate potentiation and activation of GABA_ARs (Stewart et al., 2008; Ziemba et al., 2018), also abolished [³H]muscimol binding enhancement (**Figure 6E**).

We did not test the effects of mutations on KK150 action because it minimally enhances [³H]muscimol binding. However, KK150 binds to all three of the identified NS-binding sites, and may thus be a weak partial agonist or antagonist at the sites mediating NS enhancement of [³H]muscimol binding. Consistent with this prediction, KK150 inhibited enhancement of [³H]muscimol binding by 3 α 5 α P and KK148 (**Figure 8**).

Collectively, these results show that multiple NS-binding sites contribute to enhancement of [³H]muscimol affinity and that potentiating NS (3 α 5 α P) and non-potentiating NS (3 β 5 α P, KK148 and KK150) have both common and distinct sites of action. Specifically, 3 α 5 α P enhances [³H]muscimol binding through all three sites but predominantly through the intersubunit and α_1 intrasubunit sites, which we have previously shown mediate PAM-NS potentiation (Chen et al., 2019). In contrast, 3 β 5 α P and KK148 enhance [³H]muscimol binding exclusively through the α_1 and β_3 intrasubunit sites. KK150 antagonizes the effects of KK148 on [³H]muscimol binding, presumably via the intrasubunit sites, and antagonizes the effects of 3 α 5 α P, possibly via all three sites. These data indicate that NS binding to both the intersubunit and intrasubunit sites contributes to 3 α 5 α P enhancement of [³H]muscimol binding, but that only the intrasubunit binding sites contribute to the effects of 3 β 5 α P and KK148. The data (**Figure 6B–D**) are consistent with NS producing their effects by independent action at each of the binding sites and our interpretation is based on that assumption. We cannot, however, rule out the possibility of an allosteric interaction between the NS binding sites (Chen et al., 2019).

It is important to note that the [³H]muscimol binding curves in **Figure 6** are normalized to control. The raw data show that membranes containing WT receptors have 10–20-fold higher [³H]muscimol binding (B_{\max}) than membranes containing α_1 (N408A/Y411F) β_3 receptors (**Figure 7A**), whereas the B_{\max} of membranes containing α_1 (Q242L) β_3 , $\alpha_1\beta_3$ (Y284F) or α_1 (V227W) β_3 receptors was the same as for WT $\alpha_1\beta_3$ GABA_ARs (**Figure 7—source data 1**). The lower total [³H]muscimol binding observed in α_1 (N408A/Y411F) β_3 membranes is likely a consequence of decreased receptor expression. To assure that differences in NS effect between WT and α_1 (N408A/Y411F) β_3 are not due to different muscimol affinities, we examined [³H]muscimol binding at a full range of concentrations. The α_1 (N408A/Y411F) β_3 mutations did not have a significant effect on [³H]muscimol affinity (**Figure 7B**), but eliminated the modulatory effects of NS (3 α 5 α P and KK148) on [³H]muscimol affinity (**Figure 7C–D** and **Figure 7—source data 2**). To assure that the effect of α_1 (N408A/Y411F) β_3 was specific to NS, we also examined the effect of etomidate (a non-steroidal GABA_AR PAM) on muscimol affinity. Etomidate enhanced [³H]muscimol affinity in both the WT and α_1 (N408A/Y411F) β_3 receptors, indicating that the effect of these mutations are specific to NS action (**Figure 7C–D**).

3 β 5 α P increases desensitization through binding to α_1 and β_3 intrasubunit sites

To further explore the relationship between desensitization and enhancement of [³H]muscimol binding, we examined the consequences of mutations to these sites on physiological measurements of desensitization induced by NS. Again, these experiments were performed with 3 β 5 α P because it is

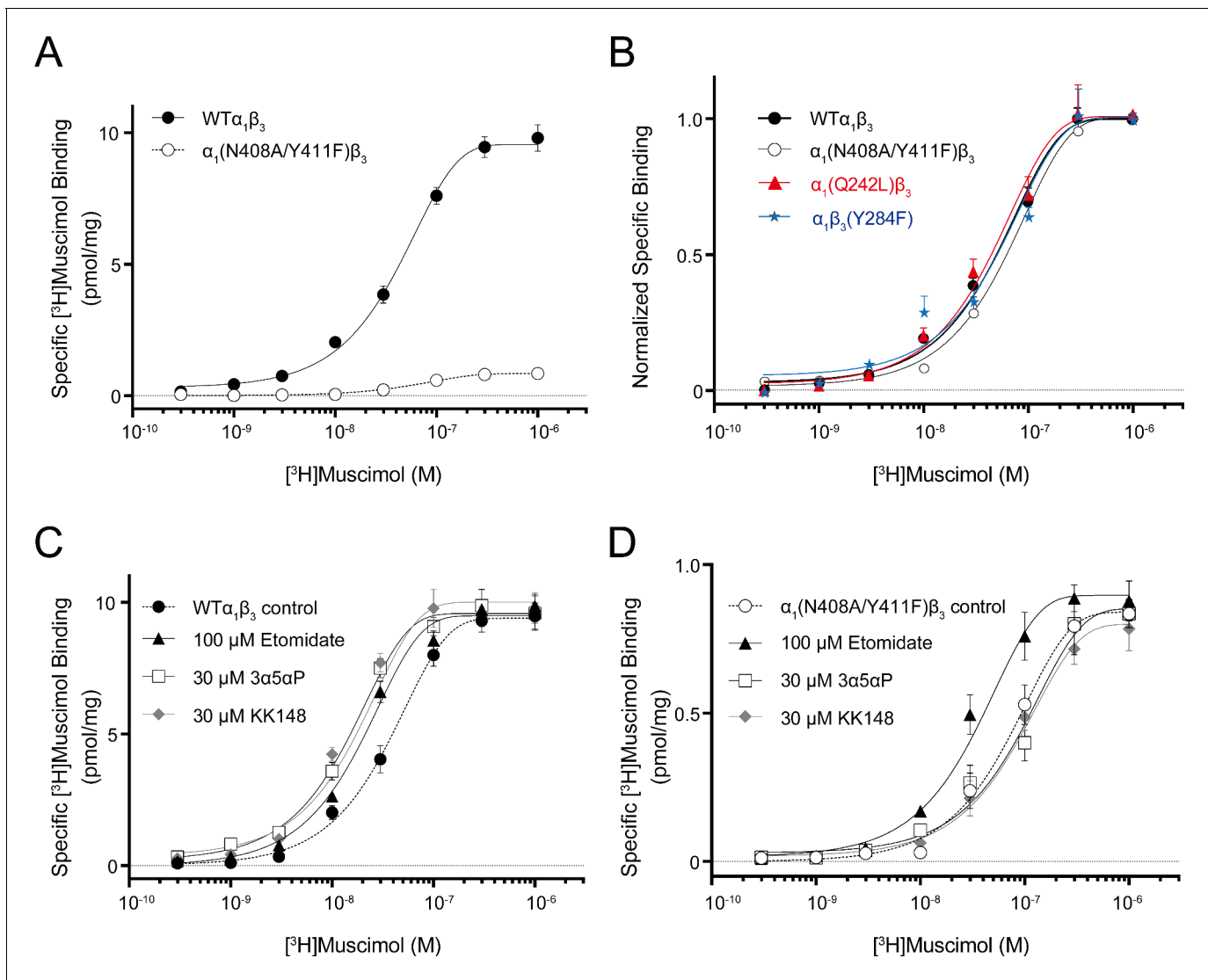


Figure 7. Neurosteroid effect on [^3H]muscimol binding isotherms in $\alpha_1\beta_3$ WT and $\alpha_1(\text{N408A/Y411F})\beta_3$ GABA $_A$ R. (A) [^3H]muscimol binding isotherms (0.3 nM–1 μM) for $\alpha_1\beta_3$ GABA $_A$ R WT and $\alpha_1(\text{N408A/Y411F})\beta_3$ GABA $_A$ R. Data points are presented as mean \pm SEM ($n = 3$). (B) Normalized curves of [^3H]muscimol binding isotherms (0.3 nM–1 μM) for $\alpha_1\beta_3$ GABA $_A$ R WT and representative mutated receptors for each neurosteroid binding site [i.e. $\alpha_1(\text{Q242L})\beta_3$ for the β_3 – α_1 intersubunit site; $\alpha_1(\text{N408A/Y411F})\beta_3$ for the α_1 intrasubunit site; $\alpha_1\beta_3(\text{Y284F})$ for the β_3 intrasubunit site]. Each data point represents mean \pm SEM ($n = 6$ for WT; $n = 3$ for mutated receptors). (C) Effect of 100 μM etomidate, 30 μM allopregnanolone (3 α 5 α P) and 30 μM KK148 on [^3H]muscimol binding isotherms in the $\alpha_1\beta_3$ GABA $_A$ R WT. (D) Same as (C) in the $\alpha_1(\text{N408A/Y411F})\beta_3$ mutant. Each data point represents mean \pm SEM ($n = 3$).

The online version of this article includes the following source data and figure supplement(s) for figure 7:

Source data 1. Properties of [^3H]muscimol binding isotherms in $\alpha_1\beta_3$ WT and mutant GABA $_A$ R.

Source data 2. Properties of neurosteroid effect on [^3H]muscimol binding isotherms in $\alpha_1\beta_3$ GABA $_A$ R WT and $\alpha_1(\text{N408A/Y411F})\beta_3$ mutant.

Figure supplement 1. Total, nonspecific and specific [^3H]muscimol binding curves.

the endogenous 3 β -OH NS and because of limited quantities of KK148. Desensitization was quantified by defining the baseline steady-state current at 1 mM GABA as 100% and measuring percent reduction of the steady-state current elicited by a NS (**Figure 9A** inset). While GABA was at a saturating concentration for all receptors, the peak P_{open} it elicited was $\ll 1.0$ for several of the mutated receptors. To normalize P_{open} , mutated receptors with low peak P_{open} were activated by co-application of 1 mM GABA with 40 μM pentobarbital (PB) (**Steinbach and Akk, 2001**) prior to application of 3 α 5 α P. This was done because the magnitude of negative allosteric modulation varies as a function of P_{open} (**Germann et al., 2019a**). To ensure that PB did not influence NS negative allosteric

Table 1. Effects of mutations on neurosteroid modulation of [3 H]muscimol binding. EC₅₀, Hill slope and maximal effect values [E_{\max} (% of control): 100% means no effect] for the concentration-response curves in **Figure 6B–E**. Statistical differences are analyzed using one-way ANOVA with Bonferroni's multiple comparisons test (* $p < 0.05$ vs. WT; ** $p < 0.01$ vs. WT). Data are presented as mean \pm SEM.

| 3 α 5 α P | EC ₅₀ (μ M) | Hill slope | E_{\max} (% of control) | N |
|---|-----------------------------|-----------------|---------------------------|---|
| WT $\alpha_1\beta_3$ | 0.24 \pm 0.04 | 1.10 \pm 0.14 | 374.1 \pm 11.1 | 6 |
| α_1 (Q242L) β_3 | **2.66 \pm 0.51 | 1.16 \pm 0.37 | **159.8 \pm 10.9 | 6 |
| α_1 (N408A/Y411F) β_3 | **2.30 \pm 0.48 | 0.87 \pm 0.44 | **146.0 \pm 9.3 | 6 |
| $\alpha_1\beta_3$ (Y284F) | 0.19 \pm 0.04 | 0.87 \pm 0.16 | 342.3 \pm 13.9 | 6 |
| α_1 (Q242L/N408A/Y411F) β_3 | - | - | **105.9 \pm 7.3 | 6 |
| 3β5αP | | | | |
| WT $\alpha_1\beta_3$ | 0.25 \pm 0.08 | 0.84 \pm 0.23 | 195.1 \pm 6.7 | 3 |
| α_1 (Q242L) β_3 | 0.27 \pm 0.09 | 0.77 \pm 0.21 | 204.3 \pm 4.5 | 3 |
| α_1 (N408A/Y411F) β_3 | 0.61 \pm 0.26 | 2.25 \pm 0.92 | **124.3 \pm 2.6 | 3 |
| $\alpha_1\beta_3$ (Y284F) | - | - | **148.6 \pm 4.9 | 3 |
| KK148 | | | | |
| WT $\alpha_1\beta_3$ | 2.40 \pm 0.36 | 1.36 \pm 0.16 | 431.0 \pm 19.5 | 6 |
| α_1 (Q242L) β_3 | 2.20 \pm 0.31 | 1.24 \pm 0.12 | 434.5 \pm 5.6 | 6 |
| α_1 (N408A/Y411F) β_3 | 1.63 \pm 0.53 | 0.73 \pm 0.23 | **161.7 \pm 3.5 | 6 |
| α_1 (V227W) β_3 | 1.73 \pm 0.68 | 0.76 \pm 0.31 | **209.2 \pm 7.4 | 6 |
| $\alpha_1\beta_3$ (Y284F) | 1.79 \pm 0.44 | 1.35 \pm 0.13 | **357.2 \pm 8.1 | 6 |
| Etomidate | | | | |
| WT $\alpha_1\beta_3$ | 7.24 \pm 1.18 | 1.07 \pm 0.17 | 331.1 \pm 9.9 | 6 |
| α_1 (Q242L) β_3 | 7.50 \pm 0.95 | 1.35 \pm 0.20 | **277.8 \pm 10.9 | 6 |
| α_1 (N408A/Y411F) β_3 | 9.14 \pm 2.20 | 1.07 \pm 0.26 | **268.2 \pm 5.9 | 6 |
| $\alpha_1\beta_3$ (Y284F) | 7.71 \pm 1.10 | 0.90 \pm 0.11 | 303.5 \pm 5.8 | 6 |
| $\alpha_1\beta_3$ (M286W) | *22.5 \pm 6.17 | 0.50 \pm 0.16 | **128.6 \pm 7.8 | 6 |

modulation, control experiments were performed in WT $\alpha_1\beta_3$ GABA_ARs and showed no significant difference in the desensitization elicited by 3 β 5 α P between receptors activated by GABA vs. GABA plus PB. The maximum P_{open} for the mutant receptors varied between 0.55 and 0.95; some of the NS effects on macroscopic currents may be influenced by these differences.

3 β 5 α P reduced the steady-state current (i.e. enhanced desensitization) by 23.0 \pm 2.8% (% of desensitization: mean \pm SEM, n = 5, **Figure 9A**). Mutations in the α_1 and β_3 intrasubunit sites [i.e. α_1 (N408A/Y411F) β_3 and $\alpha_1\beta_3$ (Y284F), respectively] prevented 3 β 5 α P-enhanced desensitization by ~67% (**Figure 9B**), whereas mutation in the β (+)- α (-) intersubunit site [α_1 (Q242L) β_3] was without effect (**Figure 9B**). Receptors with mutations in both the α_1 and β_3 intrasubunit sites [α_1 (N408A/Y411F) β_3 (Y284F)] showed less NS-enhancement of desensitization than receptors with mutations in either of the intrasubunit sites alone, indicating that both intrasubunit sites contribute to the desensitizing effect (**Figure 9B**). Although the desensitizing effect of 3 β 5 α P is completely eliminated by the V2'S mutation α_1 (V256S) β_3 , it is not completely eliminated by combined mutations of all three binding sites [α_1 (Q242L/N408A/Y411F) β_3 (Y284F)] (**Figure 9B**). This suggests either that the effects of the mutations are incomplete or there are additional unidentified NS-binding sites contributing to desensitization. Since mutations of the α_1 and β_3 intrasubunit sites also disrupt 3 β 5 α P-enhancement of [3 H]muscimol binding (**Figure 6C**), we conclude that 3 β 5 α P binding to these intrasubunit sites stabilizes the desensitized state of the GABA_AR and thus enhances [3 H]muscimol binding. Furthermore, KK148 increased GABA_AR desensitization (% of desensitization = 27.2 \pm 6.0: mean \pm SEM, n = 3, **Figure 2A**) and the α_1 (V256S) β_3 mutation abolished the effect (% of desensitization = 0, n = 1). These observations support the idea that binding of certain NS analogues to α_1 and β_3 intrasubunit

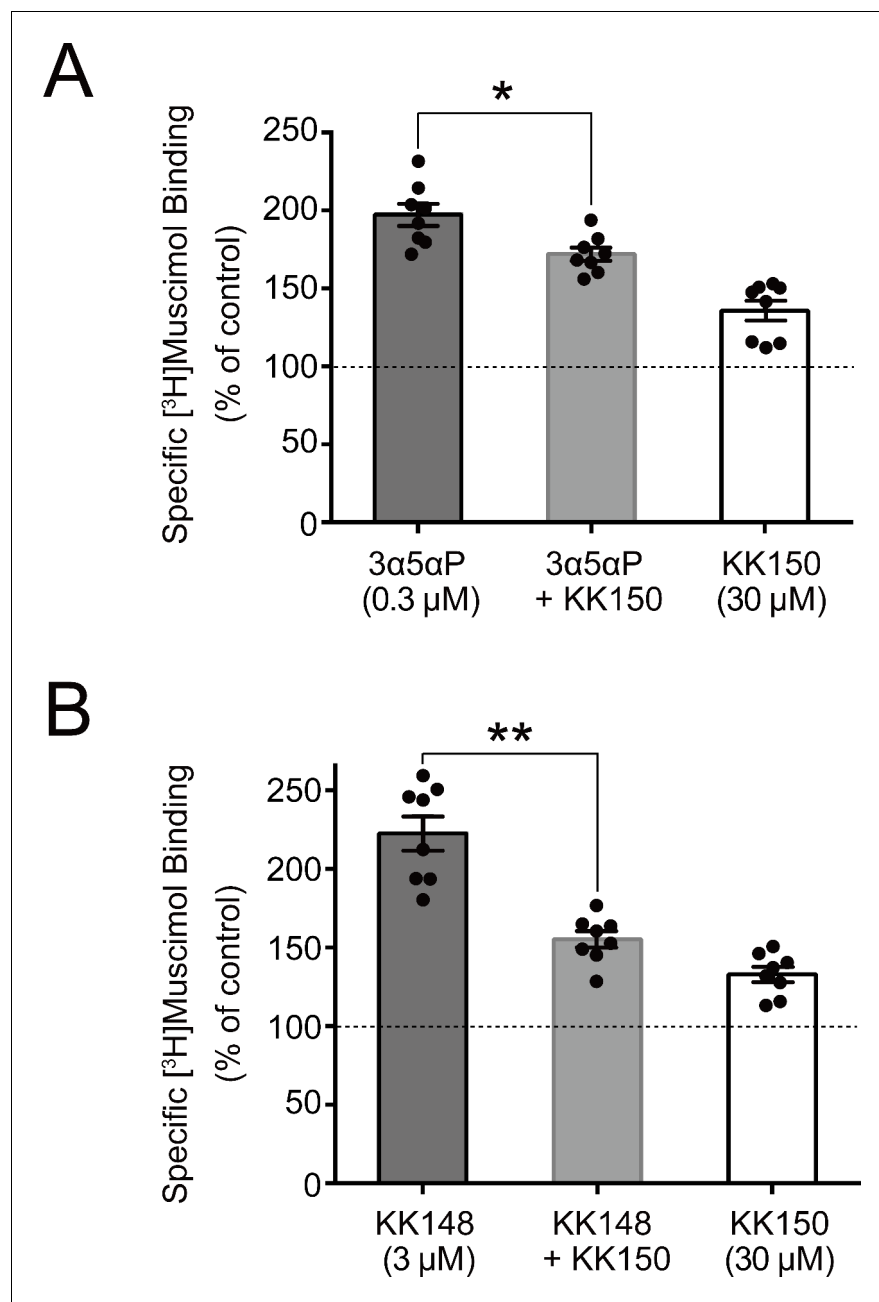


Figure 8. KK150 prevents neurosteroid-induced muscimol binding enhancement. (A) Enhancement of specific [³H] muscimol (3 nM) binding to $\alpha_1\beta_3$ GABA_AR by 0.3 μ M allopregnanolone (3 α 5 α P) in the absence (black bar) or presence (grey bar) of 30 μ M KK150 and KK150 alone (white bar). Statistical differences are analyzed using one-way ANOVA with Bonferroni's multiple comparisons test ($n = 8, \pm$ SEM). * $p < 0.05$ vs. 0.3 μ M 3 α 5 α P alone. (B) Same as (A) for 3 μ M KK148 ($n = 8, \pm$ SEM). ** $p < 0.01$ vs. 3 μ M KK148 alone.

sites, increases GABA_AR desensitization. In contrast, KK150 showed a very small effect on desensitization (% of desensitization = 2.1 ± 0.7 : mean \pm SEM, $n = 5$, **Figure 2B**), consistent with the small increase in [³H]muscimol binding by KK150 (**Figure 1E**).

The effects of 3 α 5 α P binding to intrasubunit sites on desensitization

3 α 5 α P binds to all three of the NS-binding sites on $\alpha_1\beta_3$ GABA_AR, and mutations in all three sites reduce 3 α 5 α P enhancement of [³H]muscimol binding (**Figure 6B**). This suggests the possibility that activation by 3 α 5 α P (mediated primarily by the $\beta_3(+)$ – $\alpha_1(-)$ intersubunit site) masks a desensitizing

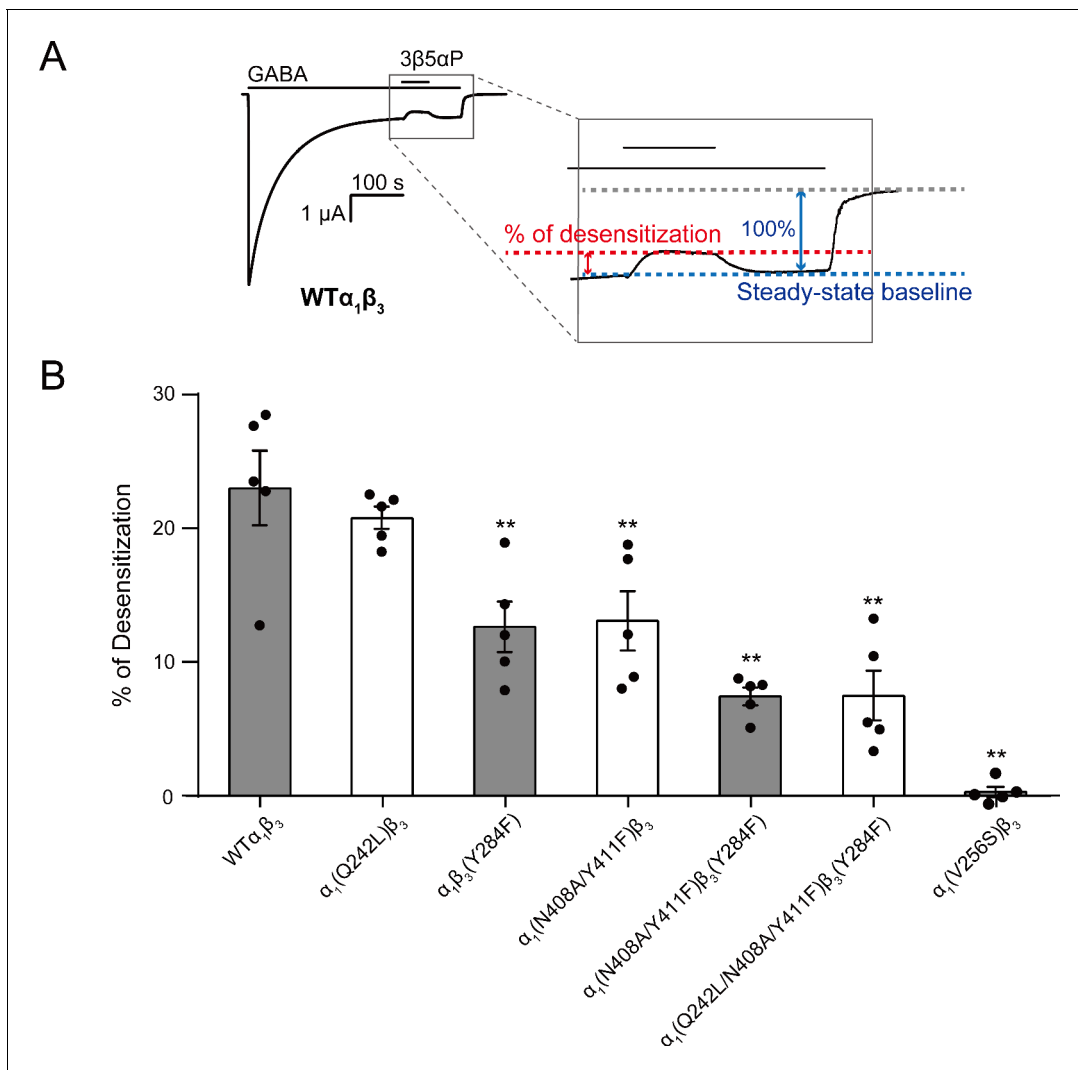


Figure 9. Mutations in intrasubunit sites prevent desensitization by epi-allopregnanolone. (A) Sample current trace showing the effect of 3 μM epi-allopregnanolone (3β5αP) on steady-state current elicited by continuous administration of 1 mM GABA to α₁β₃ GABA_AR expressed in oocytes. A zoomed-in box shows neurosteroid-induced desensitization of the steady-state GABA current. (B) Percent desensitization of the steady-state α₁β₃ GABA_AR currents (WT and mutants) by 3 μM 3β5αP during continuous application of 1 mM GABA [for WT, α₁(Q242L)β₃, α₁β₃(Y284F), α₁(N408A/Y411F)β₃ and α₁(V256S)β₃ GABA_ARs] or 1 mM GABA + 25 μM pentobarbital (PB) [for α₁(N408A/Y411F)β₃(Y284F) and α₁(Q242L/N408A/Y411F)β₃(Y284F) GABA_ARs]. The combination of GABA and PB is essential for some mutated receptors to obtain a high, consistent peak open probability. Statistical differences are analyzed using one-way ANOVA with Bonferroni's multiple comparisons test ($n = 5, \pm$ SEM). ** $p < 0.01$ vs. WT.

effect mediated through the β₃ and/or α₁ intrasubunit binding sites. To determine whether intrasubunit binding sites mediate increased desensitization by 3α5αP, we examined the effect of 3α5αP on steady-state currents in receptors with mutations in the α₁ or β₃ intrasubunit site. Mutations in the intrasubunit sites were prepared with a background α₁(Q242L)β₃ mutation to remove 3α5αP activation (Chen et al., 2019; Sugawara et al., 2019; Akk et al., 2008; Bracamontes and Steinbach, 2009) and focus on the effects of 3α5αP on the equilibrium between the open and desensitized states.

3α5αP produced a small reduction in steady-state current in α₁(Q242L)β₃ receptors with mutations in neither of the intrasubunit sites (Figure 10A). This inhibitory effect was eliminated by α₁(V256S)β₃, indicating that it was due to receptor desensitization (Figure 10D). In receptors with combined mutations in the intersubunit and α₁ intrasubunit sites [i.e. α₁(Q242L/N408A/Y411F)β₃], 3α5αP significantly inhibited the steady-state current (Figure 10B), an effect that was markedly reduced by mutations in the β₃ intrasubunit site [α₁(Q242L)β₃(Y284F)] (Figure 10C). These data

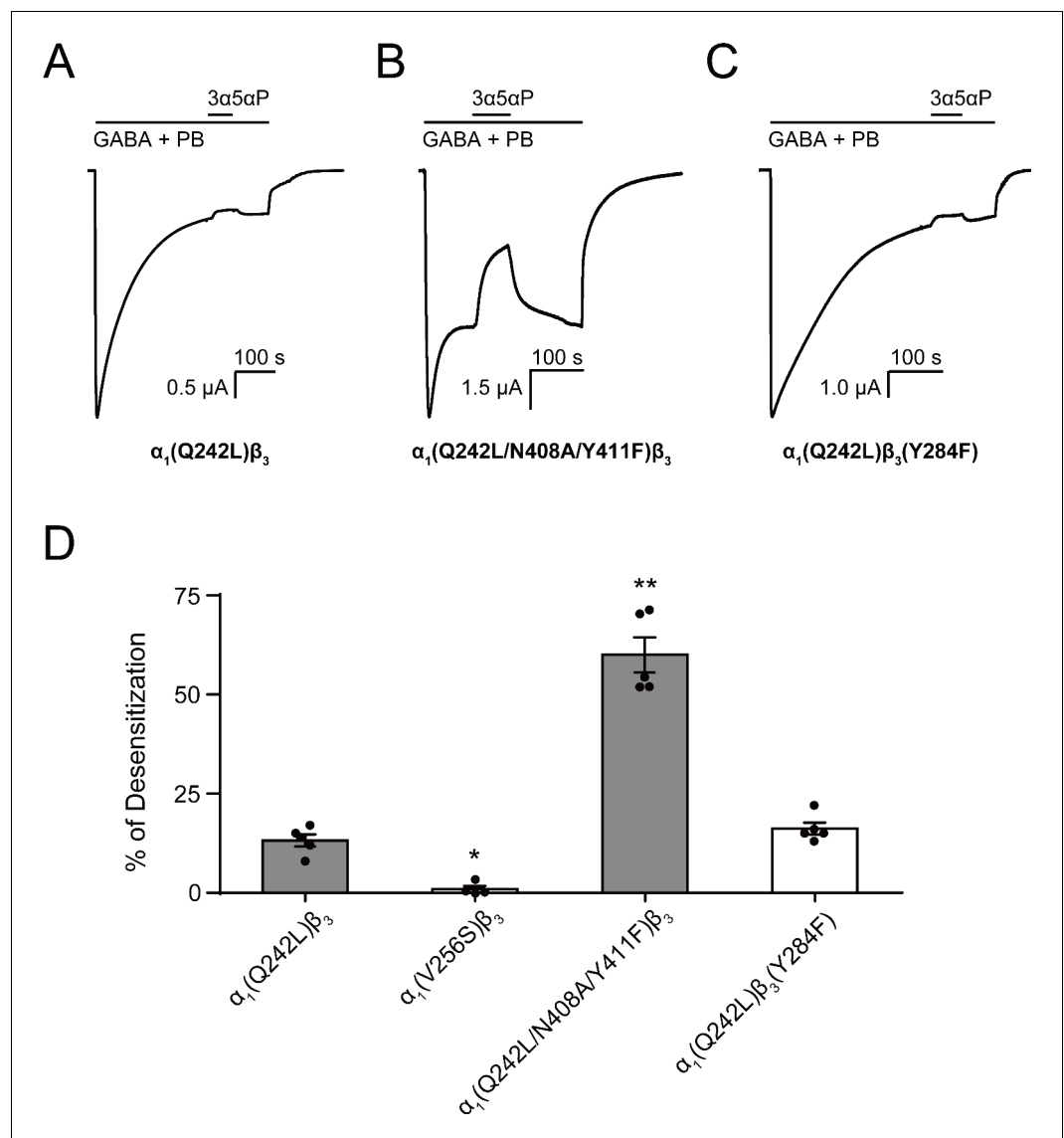


Figure 10. Allopregnanolone desensitizes GABA_AR currents via binding to the β_3 intrasubunit site. (A) Sample current trace showing the effect of 3 μ M allopregnanolone (3 α 5 α P) on $\alpha_1(Q242L)\beta_3$ GABA_AR activated by 1 mM GABA co-applied with 40 μ M pentobarbital (PB). (B), (C) Same as (A) for $\alpha_1(Q242L/N408A/Y411F)\beta_3$ GABA_AR and $\alpha_1(Q242L)\beta_3(Y284F)$ GABA_AR, respectively. (D) Percent desensitization of the steady-state currents elicited by 1 mM GABA with 40 μ M PB in $\alpha_1\beta_3$ GABA_AR with specified mutations. Statistical differences are analyzed using one-way ANOVA with Bonferroni's multiple comparisons test [$n = 4$ for $\alpha_1(V256S)\beta_3$; $n = 5$ for others, \pm SEM]. * $p < 0.05$; ** $p < 0.01$ vs. $\alpha_1(Q242L)\beta_3$, respectively.

suggest that 3 α 5 α P exerts a desensitizing effect by binding to the β_3 intrasubunit site and that 3 α 5 α P binding to the α_1 intrasubunit site does not promote desensitization (Figure 10D). Notably, 3 α 5 α P exerted only a modest inhibitory effect in $\alpha_1(Q242L)\beta_3$ receptors in which occupancy of the β_3 intrasubunit site should promote inhibition. This may be due to a counterbalancing action at the α_1 intrasubunit site, where 3 α 5 α P binding contributes more to receptor activation as demonstrated by our previous observation that mutations in the α_1 intrasubunit site significantly reduce 3 α 5 α P potentiation of GABA-elicited currents (Chen et al., 2019). These results suggest that in addition to activation, 3 α 5 α P enhances receptor desensitization. Enhanced desensitization by the PAM-NS 3 α 5 α P (Haage and Johansson, 1999) and 3 α 5 α -THDOC (Zhu and Vicini, 1997; Bianchi and MacDonald, 2003) has been observed in prior studies supporting the current finding with 3 α 5 α P.

Discussion

In this study, we examined how site-specific binding to the three identified NS sites on $\alpha_1\beta_3$ GABA_AR (Chen et al., 2019) contributes to the PAM vs. NAM activity of epimeric 3-OH NS. We found that the PAM-NS 3 α 5 α P, but not the NAM-NS 3 β 5 α P, binds to the canonical $\beta_3(+)$ – $\alpha_1(-)$ intersubunit site that mediates receptor potentiation, explaining the absence of 3 β 5 α P PAM activity. In contrast, 3 β 5 α P binds to intrasubunit sites in the α_1 and β_3 subunits, promoting receptor desensitization. Binding to the intrasubunit sites provides a mechanistic explanation for the NAM effects of 3 β 5 α P (Wang et al., 2002). 3 α 5 α P also binds to the β_3 intrasubunit site explaining the previously described desensitizing effect of the PAM-NS 3 α 5 α P (Haage and Johansson, 1999) and 3 α 5 α -THDOC (Zhu and Vicini, 1997; Bianchi and Macdonald, 2003). Two synthetic NS with diazirine moieties at C3 (KK148 and KK150) were used to identify NS-binding sites and shown to bind to the intersubunit as well as both intrasubunit sites. Neither of these ligands potentiated agonist-activated GABA_AR currents, reinforcing the importance of the 3 α -OH group and its interaction with α_1 Q242 in PAM actions. KK148 is an efficacious desensitizing agent, acting through the α_1 and β_3 intrasubunit NS-binding sites. KK150, the 17 α -epimer of KK148, binds to all three NS-binding sites, but neither activates nor desensitizes GABA_ARs, suggesting a potential chemical scaffold for a general NS antagonist. Collectively, these data show that differential occupancy of and efficacy at three discrete NS-binding sites determines whether a NS ligand has PAM, NAM, or potentially NS antagonist activity on GABA_ARs.

The observation that 3 β 5 α P and KK148 enhance orthosteric ligand binding but do not potentiate GABA-elicited currents first suggested that these NAM-NS selectively stabilize a non-conducting state that has high affinity to the orthosteric agonist muscimol. This liganded/closed state could represent a pre-active (Gielen and Corringer, 2018) or a desensitized conformation of the receptor (n.b. there may be multiple desensitized conformations of the receptor, possibly including NS-specific desensitized states). Chang and colleagues have shown that orthosteric ligand affinity (muscimol or GABA) is greater in desensitized and activated (open) GABA_ARs than in resting (closed) receptors, with estimated GABA K_d values of 78.5 μ M, 120 nM and 40 nM for the resting, activated and desensitized $\alpha_1\beta_2\gamma_2$ receptors respectively (Chang et al., 2002). Kinetic models also predict that a pre-active state should have higher affinity for an orthosteric agonist than the resting state (Gielen and Corringer, 2018). To distinguish between stabilization of a pre-active and a desensitized state, we co-applied 3 β 5 α P with a high concentration of GABA. 3 β 5 α P reduced the desensitization time constant but did not reduce peak current amplitude. While this result is consistent with stabilization of a desensitized rather than a pre-active state, it is ambiguous as it could also be explained by NAM-NS having a slower onset of effect than GABA because of slow access of the steroid to its binding site (Li et al., 2007). However, this result is supported by studies examining inhibitory postsynaptic currents (IPSCs) in which a NAM-NS can be pre-applied and the GABA concentration step is extremely rapid as well as by single channel studies. In cultured hippocampal neurons, the NAM-NS 3 β 5 β -THDOC significantly reduced IPSCs decay times but had no effect on IPSCs amplitude, consistent with a desensitizing effect (Wang et al., 2002). Single channel analyses provide the most definitive distinction between a pre-active and a desensitized state. Desensitization is predicted to shorten single channel clusters without affecting intracluster open or closed time distributions. In contrast, steroid-induced stabilization of a pre-active non-conducting state may be expected to lead to increased mean intracluster closed time. Single-channel studies examining the kinetic effects of the inhibitory steroids pregnenolone sulfate (Akk et al., 2001) or the 3 β 5 α P analogue (3 β ,5 α ,17 β)-3-hydroxyandrostane-17-carbonitrile (3 β 5 α -ACN) (Akk, G.; unpublished data) have indeed observed reduced mean cluster duration with minimal changes in intracluster open and closed time properties, indicative of the steroids promoting receptor desensitization. The α_1 V256S mutation eliminated the PS-mediated reduction in cluster duration indicating that the mutation prevents PS-mediated desensitization (Akk et al., 2001). 3 β -hydroxy steroids act similarly to PS, including exhibiting sensitivity to the α_1 V256S mutation (Wang et al., 2002). Overall, the preponderance of evidence indicates that 3 β -NAM-NS such as 3 β 5 α P inhibit GABA_AR currents by stabilizing a desensitized state.

Our experimental and modeling data demonstrate that NAM-NS such as 3 β 5 α P or KK148 enhance orthosteric ligand binding by increasing the population of receptors in a desensitized state. It is, however, unclear if 3 β 5 α P or KK148 can promote transition of resting receptors directly to a desensitized state, thus bypassing channel opening. We propose that in the presence of low

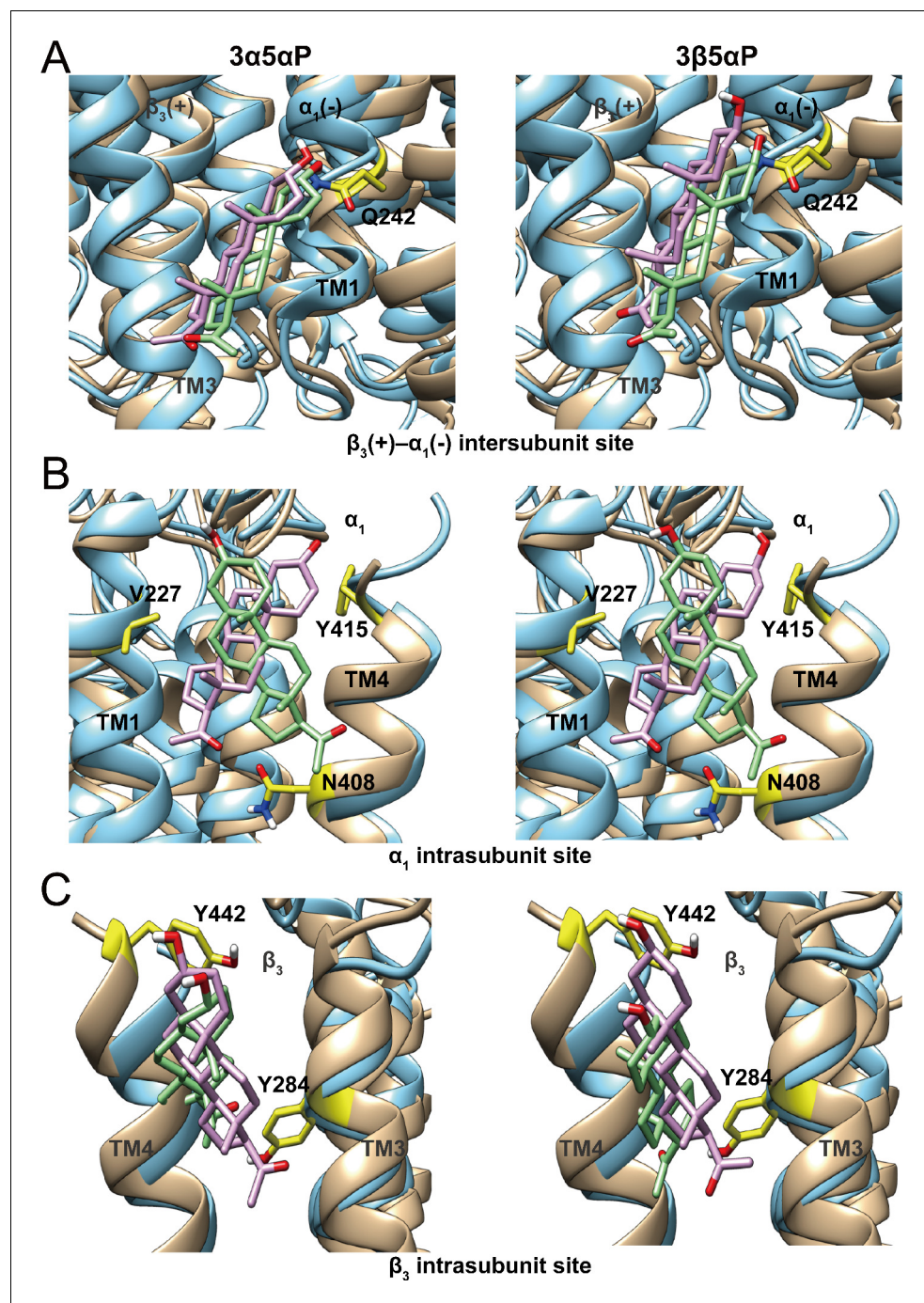


Figure 11. Comparison of allopregnanolone and epi-allopregnanolone docking poses within three neurosteroid binding pockets of the modeled $\alpha_1\beta_3$ GABA_AR TMD and the cryo-EM structure of an $\alpha_1\beta_3\gamma_2$ GABA_AR (PDB ID: 6I53). The two structures were read into UCSF Chimera and mutually aligned using MatchMaker. The $\alpha_1\beta_3$ model is shown in tan, while the $\alpha_1\beta_3\gamma_2$ structure is in cyan. (A) Representative poses for allopregnanolone (3 α 5 α P) and epi-allopregnanolone (3 β 5 α P) docked within the $\beta_3(+)$ – $\alpha_1(-)$ intersubunit site, the poses for the $\alpha_1\beta_3$ model are in pink, while those for the $\alpha_1\beta_3\gamma_2$ structure are in light green. The α_1 Q242 side chain is shown in yellow. (B) Same as for (A) for the α_1 intrasubunit site; also shown are the sidechains V227, Y415, and N408. (C) Same as (A) for the β_3 intrasubunit site; also shown are the sidechains Y284 and Y442. The Vina docking scores for 3 α 5 α P and 3 β 5 α P at each site in the $\alpha_1\beta_3$ model and the $\alpha_1\beta_3\gamma_2$ structure are shown in **Figure 11—source data 1**.

The online version of this article includes the following source data for figure 11:

Source data 1. Vina docking scores for allopregnanolone and epi-allopregnanolone at each site in $\alpha_1\beta_3$ model and $\alpha_1\beta_3\gamma_2$ GABA_AR structure.

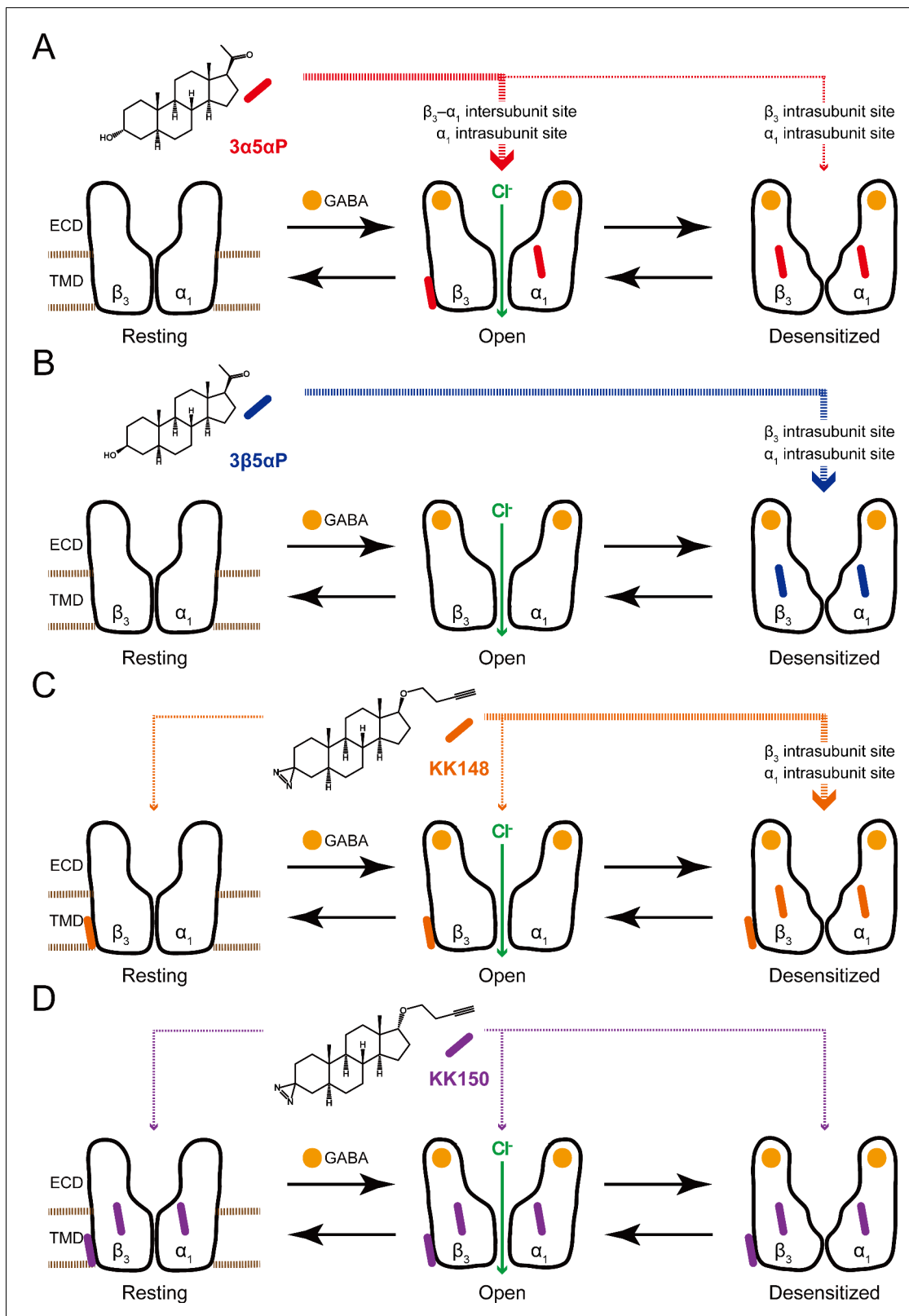


Figure 12. Neurosteroids preferentially stabilize GABA_AR in different states. (A) Model showing three fundamental conformational states that depict the channel function in the GABA_AR: a resting state; an open state; and a desensitized state. Agonist (GABA:) binding shifts the equilibrium towards high-affinity states (open and desensitized). Allopregnanolone (3α5αP:) allosterically stabilizes the high-affinity states (an open state through the β₃-α₁ intersubunit and the α₁ intrasubunit sites; a desensitized state through the β₃ intrasubunit site). The width of red arrows indicates relative affinities of

Figure 12 continued on next page

Figure 12 continued

3 α 5 α P for the open or desensitized state of the receptor. (B) Same as (A) for epi-allopregnanolone (3 β 5 α P:). 3 β 5 α P stabilizes a desensitized state through the β_3 and α_1 intrasubunit sites. (C) Same as (A) for KK148 (). KK148 allosterically stabilizes a desensitized state through the β_3 and α_1 intrasubunit sites, and equally stabilizes all three states of the receptor through the β_3 - α_1 intersubunit site. The width of orange arrows indicates relative affinities of KK148 for each state of the receptor. (D) Same as (A) for KK150 (). KK150 equally stabilizes all three states of the receptor through the β_3 and α_1 intrasubunit sites, and the β_3 - α_1 intersubunit site.

concentrations of orthosteric agonists (as in the [3 H]muscimol binding assays), there is a slow shift of receptors from resting through activated to a desensitized state with minimal change in the population of receptors in the activated state. The slow time course of accumulation of desensitized receptors is illustrated by experiments in which 10 μ M 3 β 5 α P is added to membranes that have been fully equilibrated with a low concentration (3 nM) of [3 H]muscimol and binding is measured as a function of time. Enhancement of [3 H]muscimol binding by 10 μ M 3 β 5 α P is slow, with a time constant of 4 min at 4°C ($\tau = 3.97 \pm 0.15$ min: mean \pm SEM, $n = 4$, **Figure 6—figure supplement 1**). In contrast, when $\alpha_1\beta_3$ GABA_ARs are exposed to long pulses of a high concentration of GABA, KK148- and 3 β 5 α P-induced desensitization is rapid (**Figure 2A and C**), since in these conditions almost all of the receptors are either in an open or desensitized conformation and desensitization is not slowed by the transition from resting to open state (**Jones and Westbrook, 1995**). Thus, the slow enhancement of [3 H]muscimol binding by 3 β 5 α P (**Figure 6—figure supplement 1**) is likely rate-limited by the transition of receptors to activated then to desensitized states at 3 nM muscimol rather than by 3 β 5 α P binding. These time course experiments are most consistent with a model in which receptors preferentially progress from the resting to active to desensitized states, which are then stabilized by the NAM-NS.

The selective binding of 3 β 5 α P to a subset of identified NS-binding sites provides an explanation for its NAM activity. 3 β 5 α P stabilizes desensitized receptors by binding to the α_1 and β_3 intrasubunit sites, but does not activate the receptor because it does not bind to the intersubunit site. This site-selective binding is unexpected for several reasons. First, docking and free energy perturbation calculations in a prior study predicted that 3 β 5 β P binds to the intersubunit site in a similar orientation and with free energies of binding that are equivalent to pregnanolone (3 α 5 β P) (**Miller et al., 2017**). The modeling suggested that 3 β 5 β P does not form a hydrogen bond with α Q242, a possible explanation for its lack of efficacy (**Miller et al., 2017**). Our docking studies also show similar binding energies and orientations of 3 β 5 α P and 3 α 5 α P binding in the β_3 (+)- α_1 (-) intersubunit site of either a homology model of the $\alpha_1\beta_3$ receptor or the cryo-EM structure (PDB ID: 6I53) of the $\alpha_1\beta_3\gamma_2$ receptor in a lipid nanodisc (**Laverty et al., 2019; Figure 11**). We have also shown that binding affinity or docking scores of NS binding to the intersubunit site are not significantly affected by mutations (α_1 Q242L, α_1 Q242W, α_1 W246L) that eliminate NS activation, although binding orientation is altered (**Sugasawa et al., 2019**). These data indicate that NS binding in the intersubunit site is tolerant to significant changes in critical residues and NS ligand structure, and are consistent with our findings that NS analogues, such as KK148 and KK150, can bind to the intersubunit site but have no effect on activation (**Figures 1 and 4**). Thus, the peculiar lack of 3 β 5 α P binding to the intersubunit site suggests that either: (1) details in the structure of the intersubunit site in the open conformation that explain the absence of 3 β 5 α P binding are not apparent in current high-resolution structures or; (2) 3 β 5 α P does not bind for other reasons. One plausible explanation is that 3 β 5 α P, like cholesterol, has low chemical activity in the membrane and does not achieve sufficiently available concentration to bind in this site (**Lange and Steck, 2016**). This explanation would require that the chemical activity of 3 β 5 α P differs between the inner and outer leaflets of a plasma membrane (presumably due to membrane lipid asymmetry) (**van Meer et al., 2008; Lorent et al., 2020**), since 3 β 5 α P binds to the intrasubunit sites.

The functional analysis of mutations in each of the three NS-binding sites demonstrates that the activating and desensitizing effects of NS result from occupancy of distinct sites. In particular, binding of certain NS (3 β 5 α P, KK148) to α_1 and β_3 intrasubunit sites modulates the open-desensitized equilibrium. Interestingly, lipid binding to intrasubunit pockets in bacterial pLGICs analogous to the α_1 and β_3 intrasubunit sites in GABA_AR, also modulates receptor desensitization; docosahexaenoic acid binding to an intrasubunit site in GLIC (**Basak et al., 2017**) and phosphatidylglycerol in ELIC (**Tong et al., 2019**) increase and decrease agonist-induced desensitization, respectively. The

combined results of mutational analyses and binding data demonstrate that the effects of various NS analogues are also a consequence of their efficacy at each of the sites they occupy. For example, KK148 and KK150 occupy the intersubunit site (**Figure 4C**), but do not activate GABA_AR currents (**Figure 1C–D**), and KK150 occupies both intrasubunit sites (**Figure 4B** and **Figure 4—figure supplement 1**) but does not desensitize the receptor (**Figure 2B**).

To explain the effects of the 3-substituted NS analogues, we propose a model in which NS-selective binding at three distinct binding sites on the GABA_AR preferentially stabilizes specific states (resting, open, desensitized) of the receptor (**Figure 12**). Orthosteric agonist (GABA or muscimol) binding shifts the equilibrium towards high-affinity states (open and desensitized). 3 α 5 α P allosterically stabilizes the open state through binding to the β_3 – α_1 intersubunit and α_1 intrasubunit sites and stabilizes the desensitized state through the β_3 intrasubunit site (**Figure 12A**). In contrast, 3 β 5 α P preferentially stabilizes the desensitized state through binding to both intrasubunit sites (**Figure 12B**). KK148, like 3 β 5 α P, stabilizes the desensitized state by binding to the intrasubunit sites (**Figure 12C**). KK148 also binds to the intersubunit site, presumably with no state-dependence, since it is neither an agonist nor an inverse-agonist (**Figure 1C** and **Figure 12C**). KK150, which neither activates nor desensitizes GABA_ARs and is not an inverse agonist, binds to all three sites, again presumably with no-state dependence (**Figure 1D** and **Figure 12D**). This model predicts that KK148 should act as a competitive antagonist to PAM-NS at the intersubunit site. This model also predicts that KK150 should be a competitive NS antagonist at all three binding sites. Consistent with this prediction, KK150 antagonizes 3 α 5 α P and KK148 enhancement of [³H]muscimol binding (**Figure 8**).

The site-specific model of NS action (**Figure 12**) has significant implications for the synaptic mechanisms of PAM-NS action. At a synapse, GABA_ARs are transiently exposed to high (mM) concentrations of GABA leading to a channel P_{open} approaching one (**Farrant and Nusser, 2005; Feng and Forman, 2018**). GABA is quickly cleared from the synapse leading to rapid deactivation with minimal desensitization (**Jones and Westbrook, 1995; Overstreet et al., 2000**). In the presence of a PAM-NS, deactivation is slowed, resulting in a prolongation of the IPSC and increased inhibitory current (**Harrison et al., 1987a; Zhu and Vicini, 1997; Harrison et al., 1987b; Chakrabarti et al., 2016**). This effect is largely attributable to stabilization of the open state, presumably by binding to the intersubunit and α_1 intrasubunit binding sites. A second effect has been observed in which the PAM-NS 3 α 5 α P (**Haage and Johansson, 1999**) and 3 α 5 α -THDOC (**Zhu and Vicini, 1997**) prolong the slow component of GABA_AR desensitization and slow recovery from desensitization. This results in increased late channel openings (**Zhu and Vicini, 1997; Jones and Westbrook, 1995**) and IPSC prolongation (**Harrison et al., 1987b; Chakrabarti et al., 2016**). When the frequency of synaptic firing is rapid, the desensitizing effect of NS may also contribute to frequency-dependent reduction in IPSC amplitude (**Zhu and Vicini, 1997; Jones and Westbrook, 1996**). The desensitizing effect of 3 α 5 α P is predominantly mediated by binding at the β_3 intrasubunit site. The balance between stabilization of the open and desensitized channels should be determined by the relative occupancies for the intersubunit site of the active receptor and the β_3 intrasubunit site of the desensitized receptor. Computational docking of 3 α 5 α P to these sites indicates modest differences in affinity between the sites with a rank order affinity of: intersubunit > α_1 intrasubunit > β_3 intrasubunit sites (**Figure 11—source data 1; Chen et al., 2019**). Mutational analysis of the effects of NS on enhancement of [³H]muscimol binding also indicates that 3 α 5 α P has a lower affinity to the β_3 intrasubunit site (**Figure 6B, Table 1**). Thus, binding to the β_3 intrasubunit site may serve as a negative feedback mechanism preventing excessive PAM-NS effects on synaptic currents.

We have identified specific NS-binding sites on α_1 and β_3 GABA_ARs using photoaffinity labeling, but the structural details of NS interactions with these sites have not yet been elucidated. While several high-resolution structures of $\alpha\beta\gamma$ GABA_ARs in a lipidic environment (nano-discs) with bound orthosteric or allosteric ligands have been published (**Laverty et al., 2019; Masiulis et al., 2019; Kim et al., 2020**), there are no available structures of a heteropentameric GABA_AR with a bound NS. There are, however, three X-ray structures of homopentameric, chimeric GABA_ARs crystallized from detergent, all showing NS bound only in the intersubunit site (**Miller et al., 2017; Laverty et al., 2017; Chen et al., 2018**). Since we identified intrasubunit NS-binding sites by photo-labeling full-length heteropentameric GABA_ARs in native membranes, it is possible that either the non-natural ECD-TMD junctions or the detergent environment explain why neurosteroid binding to an intrasubunit site was not observed. However, we have also identified an α_1 intrasubunit NS

binding site using a 3 α 5 α P analogue photolabeling reagent in a detergent-solubilized ELIC- α_1 chimeric receptor (Sugasawa *et al.*, 2019), indicating that NS can bind to this site in a detergent-solubilized chimeric receptor. It is important to note that there are many reasons why a bound NS (or any other ligand) may not be observed in X-ray or cryo-EM structures and that while these methods provide exquisite detail regarding the structure of ligand binding sites, they should not be regarded as a litmus test for the identification of ligand-binding sites. These reasons include: (1) a protein with steroid bound in the intrasubunit site may not form good crystals or may aggregate, precluding single particle analysis. (2) The conformation(s) observed in the X-ray or cryo-EM structure may not be the conformation to which NS preferentially binds in the intrasubunit site. (3) The ECD-TMD junction is the area of the protein that undergoes the most movement with activation and can be the least well-resolved portion of the transmembrane domains. (4) NS have multiple binding orientations and may be more mobile within the intrasubunit site making them more difficult to resolve. It is also important to consider that our analyses of the functional effects of NS binding were performed using $\alpha_1\beta_3$ GABA_ARs. While the actions of inhibitory and potentiating NS in $\alpha\beta\gamma$ or $\alpha\beta\delta$ receptors are qualitatively similar to those observed in $\alpha\beta$ receptors, the trimeric receptors may have one less intrasubunit NS-binding site per pentamer, or an intrasubunit with different NS specificity or effect. Additionally, the presence of a γ -subunit has been shown to alter the conformational symmetry of the GABA_AR (Lavery *et al.*, 2019; Kim *et al.*, 2020; Zhu *et al.*, 2018) and may influence NS binding to an intrasubunit site or its functional effects.

In summary, this study describes a unique NS pharmacology in which different NS analogues selectively bind to subsets of three sites on the $\alpha_1\beta_3$ GABA_AR, with each analogue exhibiting state-dependent binding at a given site. The combination of site-selectivity and state-dependence of binding determines whether a NS analogue is a PAM, a NAM or an antagonist of NS action at the GABA_AR. It seems likely that other GABA_AR subunit isoforms and heteropentameric subunit combinations will reveal additional NS-binding sites with distinct affinity and state-dependence for various analogues. The identification of potent agonists and antagonists for each of these sites will provide tools for understanding the biological effects of endogenous neurosteroids and potentially for the development of precision neurosteroid therapeutics.

Materials and methods

Construct design

The human α_1 and β_3 GABA_AR subunits were subcloned into pcDNA3 for molecular manipulations and cRNA synthesis. Using QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA), a FLAG tag was first added to the α_1 subunit then an 8xHis tag was added to generate the following His-FLAG tag tandem (QPSLHHHHHHHHHDYKDDDDKDEL), inserted between the fourth and fifth residues of the mature peptide. The α_1 and β_3 subunits were then transferred into the pcDNA4/TO and pcDNA5/TO vectors (Thermo Fisher Scientific), respectively, for tetracycline inducible expression. Transient expression was done using the GABA_AR subunits rat α_1 FLAG (Ueno *et al.*, 1996) and human β_3 obtained from Geoffrey White (Neurogen, Branford, CT), each were subcloned into pcDNA3 for molecular manipulations and cRNA synthesis. Point mutations were generated using the QuikChange Site-Directed Mutagenesis Kit (Agilent) and the coding region was fully sequenced prior to use. The cDNAs were linearized with XbaI (NEB Labs, Ipswich, MA), and the cRNAs were generated using T7 mMessage mMachine (Ambion, Austin, TX).

Cell lines

Cell culture was performed as described in previous reports (Chen *et al.*, 2019). The tetracycline inducible cell line T-REx-HEK293 (Thermo Fisher Scientific) was cultured under the following conditions: cells were maintained in DMEM/F-12 50/50 medium containing 10% fetal bovine serum (tetracycline-free, Takara, Mountain View, CA), penicillin (100 units/ml), streptomycin (100 g/ml), and blasticidin (2 μ g/ml) at 37°C in a humidified atmosphere containing 5% CO₂. Cells were passaged twice each week, maintaining subconfluent cultures. Stably transfected cells were cultured as above with the addition of hygromycin (50 μ g/ml) and zeocin (20 μ g/ml). A stable cell line was generated by transfecting T-REx-HEK293 cells with human α_1 -8xHis-FLAG pcDNA4/TO and human β_3 pcDNA5/TO, in a 150 mm culture dish, using the Effectene transfection reagent (Qiagen, Germantown, MD).

Two days after transfection, selection of stably transfected cells was performed with hygromycin and zeocin until distinct colonies appeared. Medium was exchanged several times each week to maintain antibiotic selection. Individual clones were selected from the dish and transferred to 24-well plates for expansion of each clone selected. When the cells grew sufficiently, about 50% confluence, they were split into two other plates, one for a surface ELISA against the FLAG epitope and a second for protein assay, to normalize surface expression to cell number. The best eight clones were selected for expansion into 150 mm dishes, followed by [3 H]muscimol binding to examine the receptor density. Once the best expressing clone was determined, the highest expressing cells of that clone were selected through fluorescence activated cell sorting. Transient transfections were done in HEK293S GnTI⁻ cells obtained from ATCC (CRL-3022) using Effectene (Qiagen). The identity of the cell lines has been authenticated using short tandem repeat analysis. Mycoplasma test performed on the cells used for these experiments was negative.

Membrane protein preparation

Stably transfected cells were plated into dishes. After reaching 50% confluence, GABA_A receptors were expressed by inducing cells with 1 μ g/ml of doxycycline with the addition of 5 mM sodium butyrate. Cells were harvested 48 to 72 hr after induction. HEK cells, after induction, were grown to 100% confluence, harvested and washed with 10 mM potassium phosphate, 100 mM potassium chloride (pH 7.5) plus protease inhibitors (Sigma-Aldrich, St. Louis, MO) two times. The cells were collected by centrifugation at 1,000 g at 4°C for 5 min. The cells were homogenized with a glass mortar and a Teflon pestle for ten strokes on ice. The pellet containing the membrane proteins was collected after centrifugation at 20,000 g at 4°C for 45 min and resuspended in a buffer containing 10 mM potassium phosphate, 100 mM potassium chloride (pH 7.5). The protein concentration was determined with micro-BCA protein assay and membranes were stored at –80°C.

Photolabeling and purification of $\alpha_1\beta_3$ GABA_AR

The syntheses of neurosteroid photolabeling reagents (KK148, KK150, KK200, KK123) are detailed in previous reports (Jiang et al., 2016; Cheng et al., 2018). For all the photolabeling experiments, 10–20 mg of HEK cell membrane proteins (about 300 pmol [3 H]muscimol binding) were thawed and resuspended in buffer containing 10 mM potassium phosphate, 100 mM potassium chloride (pH 7.5) and 1 mM GABA at a final concentration of 1.25 mg/ml. For the photolabeling competition experiments, 3 μ M KK200 or KK123 in the presence of 30 μ M competitor (3 α 5 α P, KK148, KK150, and 3 β 5 α P) or the same volume of ethanol was added to the membrane proteins and incubated on ice for 1 hr. The samples were then irradiated in a quartz cuvette for 5 min, by using a photoreactor emitting light at >320 nm. The membrane proteins were then collected by centrifugation at 20,000 g at 4°C for 45 min. The photolabeled membrane proteins were resuspended in lysis buffer containing 1% *n*-dodecyl- β -D-maltoside (DDM), 0.25% cholesteryl hemisuccinate (CHS), 50 mM Tris (pH 7.5), 150 mM NaCl, 2 mM CaCl₂, 5 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 10% glycerol at a final concentration of 1 mg/ml. The membrane protein suspension was homogenized using a glass mortar and a Teflon pestle and incubated at 4°C overnight. The protein lysate was centrifuged at 20,000 g at 4°C for 45 min and supernatant was incubated with 0.5 ml anti-FLAG agarose (Sigma) at 4°C for 2 hr. The anti-FLAG agarose was then transferred to an empty column, followed by washing with 20 ml washing buffer (50 mM triethylammonium bicarbonate and 0.05% DDM). The GABA_ARs were eluted with aliquots of 200 μ g/ml FLAG tag peptide and 100 μ g/ml 3X FLAG (ApexBio) in the washing buffer. The pooled eluates (9 ml) containing GABA_ARs were concentrated to 100 μ l using 100 kDa cut-off centrifugal filters.

Tryptic middle-down MS analysis

The purified $\alpha_1\beta_3$ GABA_AR (100 μ l) was reduced with 5 mM tris(2-carboxyethyl)phosphine for 1 hr, alkylated with 5 mM *N*-ethylmaleimide (NEM) for 1 hr, and quenched with 5 mM dithiothreitol (DTT) for 15 min. These three steps were done at RT. Samples were then digested with 8 μ g of trypsin for 7 days at 4°C to obtain maximal recovery of TMD peptides. The digestions were terminated by adding formic acid in a final concentration of 1%, followed directly by LC-MS analysis on an Orbitrap Elite mass spectrometer. 20 μ l samples were injected into a home-packed PLRP-S (Agilent, Santa Clara, CA) column (10 cm \times 75 μ m, 300 Å), separated with a 145 min gradient from 10% to 90%

acetonitrile, and introduced to the mass spectrometer at 800 nl/min with a nanospray source. MS acquisition was set as a MS1 Orbitrap scan (resolution of 60,000) followed by top 20 MS2 Orbitrap scans (resolution of 15,000) using data-dependent acquisition, and exclusion of singly charged precursors. Fragmentation was performed using high-energy dissociation with normalized energy of 35%. Analysis of data sets was performed using Xcalibur (Thermo Fisher Scientific) to manually search for TM1, TM2, TM3 or TM4 tryptic peptides with or without neurosteroid photolabeling modifications. Photolabeling efficiency was estimated by generating extracted chromatograms of unlabeled and labeled peptides, determining the area under the curve, and calculating the abundance of labeled peptide/(unlabeled + labeled peptide). Analysis of statistical significance comparing the photolabeling efficiency of KK200 and KK123 for $\alpha_1\beta_3$ GABA_AR was determined using one-way ANOVA with Bonferroni's multiple comparisons test and paired t-test, respectively (Prism 6, GraphPad Software, San Diego, CA). MS2 spectra of photolabeled TMD peptides were analyzed by manual assignment of fragment ions with and without photolabeling modification. Fragment ions were accepted based on the presence of a monoisotopic mass within 20 ppm mass accuracy. In addition to manual analysis, PEAKS (Bioinformatics Solutions Inc, Waterloo, ON, Canada) database searches were performed for data sets of photolabeled $\alpha_1\beta_3$ GABA_AR. Search parameters were set for a precursor mass accuracy of 20 ppm, fragment ion accuracy of 0.1 Da, up to three missed cleavages on either end of the peptide, false discovery rate of 0.1%, and variable modifications of methionine oxidation, cysteine alkylation with NEM and DTT, and NS analogue photolabeling reagents on any amino acid.

Radioligand-binding assays

[³H]muscimol-binding assays were performed using a previously described method (Chen et al., 2019). HEK cell membrane proteins (100 µg/ml final concentration) were incubated with 3 nM [³H]muscimol (30 Ci/mmol; PerkinElmer Life Sciences), neurosteroid (3 nM–30 µM) or etomidate (30 nM–200 µM) in different concentrations and binding buffer (10 mM potassium phosphate, 100 mM potassium chloride, pH 7.5) in a total volume of 1 ml. Assay tubes were incubated for 1 hr on ice in the dark. Nonspecific binding was determined by binding in the presence of 1 mM GABA. Membranes were collected on Whatman/GF-C glass filter papers using a Brandel cell harvester (Gaithersburg, MD). To perform [³H]muscimol binding isotherms, 100 µg/ml aliquots of membrane protein were incubated with 0.3 nM–1 µM [³H]muscimol for 1 hr on ice in the dark. The specific activity for [³H]muscimol concentrations from 30 nM to 1 µM was reduced to 2 Ci/mmol by dilution with nonradioactive muscimol. The membranes were collected on Whatman/GF-B glass filter papers using a vacuum manifold. Raw concentration-dependent total and nonspecific binding and calculated specific binding data from a representative experiment (WT receptors, no NS) are shown in **Figure 7—figure supplement 1**. For [³H]muscimol-binding experiments examining competitive interactions between neurosteroids, the combined neurosteroids (0.3 µM 3 α 5 α P or 3 µM KK148 ±30 µM KK150) or the same volume of dimethyl sulfoxide (DMSO) were added to the membranes which were then incubated with 3 nM [³H]muscimol on ice for 1 hr. Time courses of neurosteroid [³H]muscimol binding enhancement were examined by adding 10 µM of neurosteroids (3 α 5 α P, 3 β 5 α P) to membranes that had been fully equilibrated with 3 nM [³H]muscimol for 1 hr on ice; binding was then measured as a function of time at 1, 3, 10, 30, 60 min. The membranes were collected on Whatman/GF-B glass filter papers using a vacuum manifold. Radioactivity bound to the filters was measured by liquid scintillation spectrometry using Bio-Safe II (Research Products International, Mount Prospect, IL).

Radioligand binding to intact cells

HEK cells were harvested by gently washing dishes with buffer containing 10 mM sodium phosphate (pH 7.5), 150 mM sodium chloride twice. The cells were collected by centrifugation at 500 g at 4°C for 5 min, and resuspended in isotonic (10 mM sodium phosphate, 150 mM sodium chloride, pH 7.5) or hypotonic (10 mM sodium phosphate, pH 7.5) buffer to prepare two different conditions for radioligand binding to intact cells [isotonic buffer for cell surface receptors; hypotonic buffer for total receptors (cell surface receptors + intracellular receptors)]. The cells were incubated on ice for 2 hr, after which the sodium chloride concentration was adjusted to be isotonic before the radioligand binding procedure. HEK cells were aliquoted to assay tubes (20 samples/150 mm dish) in a total volume of 1 ml, and incubated with 3 nM [³H]muscimol ±10 µM KK148 for 1 hr on ice in the dark.

Nonspecific binding was determined by binding in the presence of 1 mM GABA. The membranes were collected on Whatman/GF-B glass filter papers using a vacuum manifold. Radioactivity bound to the filters was measured by liquid scintillation spectrometry using Bio-Safe II.

Receptor expression in *Xenopus laevis* oocytes and electrophysiological recordings

The wild-type and mutant $\alpha_1\beta_3$ GABA_AR were expressed in oocytes from the African clawed frog (*Xenopus laevis*). Frogs were purchased from Xenopus 1 (Dexter, MI), and housed and cared for in a Washington University Animal Care Facility under the supervision of the Washington University Division of Comparative Medicine. Harvesting of oocytes was conducted under the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. The animal protocol (No. 20180191) was approved by the Animal Studies Committee of Washington University in St. Louis. The oocytes were injected with a total of 12 ng cRNA. The ratio of cRNAs was 5:1 ratio ($\alpha_1:\beta_3$) to minimize the expression of β_3 homomeric receptors. Following injection, the oocytes were incubated in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES; pH 7.4) with supplements (2.5 mM Na pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin) at 16°C for 2 days prior to conducting electrophysiological recordings. The electrophysiological recordings were conducted at room temperature using standard two-electrode voltage clamp. The oocytes were clamped at −60 mV. The chamber (RC-1Z, Warner Instruments, Hamden, CT) was perfused with ND96 at 5–8 ml/min. Solutions were gravity-applied from 30 ml glass syringes with glass luer slips via Teflon tubing. The current responses were amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT), digitized with a Digidata 1200 series digitizer (Molecular Devices), and stored using pClamp (Molecular Devices). Current traces were analyzed with Clampfit (Molecular Devices). Activation by steroids (**Figure 1**) was tested by coapplying a steroid with 0.3 µM GABA ($P_{\text{open}} = 0.05\text{--}0.1$). The desensitizing effects of steroids (**Figures 9–10**) were tested by coapplying a steroid with 1 mM (saturating) GABA, alone or in the presence of PB, during the steady-state phase of the current response. The combination of GABA and PB was used to activate some combinations of mutations to maintain a consistent, high peak open probability (0.55–0.95). In control experiments in WT $\alpha_1\beta_3$ GABA_ARs, pentobarbital had no effect on 3β5αP inhibition of steady-state current (data not shown). The stock solution of GABA was made in ND96 at 500 mM, stored in aliquots at −20°C, and diluted on the day of experiment. The stock solution of muscimol was made at 20 mM in ND96 and stored at 4°C. The steroids were dissolved in DMSO at 10–20 mM and stored at room temperature.

Electrophysiological data analysis and simulations

The raw amplitudes of the current traces were converted to units of open probability through comparison to the peak response to 1 mM GABA + 50 µM propofol, that was considered to have a peak P_{open} indistinguishable from 1 (**Chen et al., 2019**). The level of constitutive activity in the absence of any applied agonist was considered negligible and not included in this calculation. The converted current traces were analyzed in the framework of the three-state Resting-Open-Desensitized activation model (**Germann et al., 2019a; Germann et al., 2019b**). The model enables analysis and prediction of peak responses using four parameters that characterize the extent of constitutive activity (termed L ; $L = \text{Resting/Open}$), affinity of the resting receptor to agonist (K_C), affinity of the open receptor to agonist (K_O), and the number of agonist binding sites (N). Analysis and prediction of steady-state responses requires an additional parameter, termed Q , that describes the equilibrium between open and desensitized receptors ($Q = \text{Open/Desensitized}$).

The P_{open} of the peak response is expressed as:

$$P_{\text{open,peak}} = \frac{1}{1 + L\Gamma}$$

and the P_{open} of the steady-state response as:

$$P_{\text{open,steady-state}} = \frac{1}{1 + \frac{1}{Q} + L\Gamma}$$

where

$$\Gamma = \left[\frac{(1 + [X]/K_C)}{(1 + [X]/K_O)} \right]^N$$

[X] is the concentration of agonist present, and other terms are as described above. In practice, the value of Γ was calculated using the experimentally determined P_{open} of the peak response, and then used as a fixed value in estimating Q from $P_{\text{open, steady-state}}$.

The $P_{\text{desensitized}}$ was calculated using:

$$P_{\text{desensitized}} = \frac{1}{1 + Q + Q\Gamma}$$

The effect of $3\beta 5\alpha P$ on 1 mM GABA-elicited steady-state current was expressed through a change in the value of Q . The modified Q (termed Q^*) was then used to predict changes in P_{open} and $P_{\text{desensitized}}$ at low [GABA]. Calculated probabilities (e.g. P_{open} , $P_{\text{desensitized}}$) are reported as mean \pm SD.

Docking simulations

A model of the $\alpha_1\beta_3$ GABA_AR was developed using the crystal structure of the human β_3 homopentamer (PDB ID: 4COF) as a structural template (Miller and Aricescu, 2014). In this structure, the cytoplasmic loop was replaced with the sequence SQPARAA (Jansen et al., 2008). The pentamer subunits were organized as A α_1 , B β_3 , C α_1 , D β_3 , E β_3 . The α_1 sequence was aligned to the β_3 sequence using the program MUSCLE (Edgar, 2004). The pentameric alignment was then used as input for the program Modeller (Sali and Blundell, 1993), using 4COF as the template; a total of 25 models were generated. The best model as evaluated by the DOPE score (Shen and Sali, 2006) was then submitted to the H++ server (<http://biophysics.cs.vt.edu>) to determine charges and optimize hydrogen bonding. The optimized structure was then submitted to the PPM server (https://opm.phar.umich.edu/ppm_server) for orientation into a lipid membrane. The correctly oriented receptor was then submitted to the CHARMM-GUI Membrane Builder server (<http://www.charmm-gui.org>) to build the fully solvated, membrane bound system oriented into a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) bilayer. The system was fully solvated with 40715 TIP3 water molecules and ionic strength set to 0.15 M KCl. The NAMD input files produced by CHARMM-GUI (Lee et al., 2016) use a seven-step process of gradually loosening constraints in the simulation prior to production runs. A 100 ns molecular dynamics trajectory was then obtained using the CHARMM36 force field and NAMD (Lee et al., 2016). The resulting trajectory was then processed using the utility mdtraj (McGibbon et al., 2015), to extract a snapshot of the receptor at each nanosecond of time frame. These structures were then mutually aligned by fitting the alpha carbons, providing a set of 100 mutually aligned structures used for docking studies. The docking was performed using AutoDock Vina (Trott and Olson, 2010) on each of the 100 snapshots in order to capture receptor flexibility. $3\alpha 5\alpha P$ and $3\beta 5\alpha P$ were prepared by converting the sdf file from PubChem into a PDB file using Open Babel (O'Boyle et al., 2011), and Gasteiger charges and free torsion angles were determined by AutoDock Tools. Docking grid boxes were built for the β_3 - α_1 intersubunit, and the α_1 and β_3 intrasubunit sites with dimensions of $15 \times 15 \times 15$ Ångströms encompassing each binding pocket. Docking was limited to an energy range of 3 kcal from the best docking pose and was limited to a total of 20 unique poses. The docking results for a given site could result in a maximum of 2000 unique poses (20 poses \times 100 receptor structures); these were then clustered geometrically using the program DIVCF (Meslamani et al., 2009). The resulting clusters were ranked by Vina score and cluster size, and then visually analyzed. A comparison of proposed NS-binding sites between the modeled $\alpha_1\beta_3$ GABA_AR TMD and the experimentally determined $\alpha_1\beta_3\gamma_2$ cryo-EM structure PDB ID: 6I53 (Lavery et al., 2019) was performed. The two structures were read into UCSF Chimera and mutually aligned using MatchMaker (Meng et al., 2006). Using the same Vina docking configuration files discussed above, $3\alpha 5\alpha P$ and $3\beta 5\alpha P$ were then docked into the respective sites of the $\alpha_1\beta_3\gamma_2$ cryo-EM structure. The results are shown in Figure 11 and Figure 11—source data 1; there was very little difference in the results between the modeled $\alpha_1\beta_3$ and the $\alpha_1\beta_3\gamma_2$ cryo-EM structure.

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Author contributions

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Author ORCIDs

Yusuke Sugasawa  <https://orcid.org/0000-0003-1607-0460>

Wayland WL Cheng  <http://orcid.org/0000-0002-9529-9820>

Zi-Wei Chen  <http://orcid.org/0000-0001-8601-2210>

Alex S Evers  <https://orcid.org/0000-0002-0342-0575>

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Additional files

Supplementary files

- Transparent reporting form

Data availability

All data generated or analysed during this study are included in the manuscript and supporting files.

References

- Abramian AM**, Comenencia-Ortiz E, Modgil A, Vien TN, Nakamura Y, Moore YE, Maguire JL, Terunuma M, Davies PA, Moss SJ. 2014. Neurosteroids promote phosphorylation and membrane insertion of extrasynaptic GABA_A receptors. *PNAS* **111**:7132–7137. DOI: <https://doi.org/10.1073/pnas.1403285111>, PMID: 24778259
- Akk G**, Bracamontes J, Steinbach JH. 2001. Pregnenolone sulfate block of GABA_A receptors: mechanism and involvement of a residue in the M2 region of the alpha subunit. *The Journal of Physiology* **532**:673–684. DOI: <https://doi.org/10.1111/j.1469-7793.2001.0673e.x>, PMID: 11313438
- Akk G**, Bracamontes JR, Covey DF, Evers A, Dao T, Steinbach JH. 2004. Neuroactive steroids have multiple actions to potentiate GABA_A receptors. *The Journal of Physiology* **558**:59–74. DOI: <https://doi.org/10.1113/jphysiol.2004.066571>, PMID: 15146041
- Akk G**, Covey DF, Evers AS, Steinbach JH, Zorumski CF, Mennerick S. 2007. Mechanisms of neurosteroid interactions with GABA(A) receptors. *Pharmacology & Therapeutics* **116**:35–57. DOI: <https://doi.org/10.1016/j.pharmthera.2007.03.004>, PMID: 17524487
- Akk G**, Li P, Bracamontes J, Reichert DE, Covey DF, Steinbach JH. 2008. Mutations of the GABA-A receptor alpha1 subunit M1 domain reveal unexpected complexity for modulation by neuroactive steroids. *Molecular Pharmacology* **74**:614–627. DOI: <https://doi.org/10.1124/mol.108.048520>, PMID: 18544665
- Akk G**, Covey DF, Evers AS, Mennerick S, Zorumski CF, Steinbach JH. 2010. Kinetic and structural determinants for GABA-A receptor potentiation by neuroactive steroids. *Current Neuropharmacology* **8**:18–25. DOI: <https://doi.org/10.2174/157015910790909458>, PMID: 20808543
- Akk G**, Germann AL, Sugawara Y, Pierce SR, Evers AS, Steinbach JH. 2020. Enhancement of muscimol binding and gating by allosteric modulators of the GABA_{AA} Receptor: Relating Occupancy to State Functions. *Molecular Pharmacology* **98**:303–313. DOI: <https://doi.org/10.1124/molpharm.120.000066>, PMID: 32873746
- Basak S**, Schmandt N, Gicheru Y, Chakrapani S. 2017. Crystal structure and dynamics of a lipid-induced potential desensitized-state of a pentameric ligand-gated channel. *eLife* **6**:e23886. DOI: <https://doi.org/10.7554/eLife.23886>, PMID: 28262093
- Belelli D**, Lambert JJ. 2005. Neurosteroids: endogenous regulators of the GABA(A) receptor. *Nature Reviews Neuroscience* **6**:565–575. DOI: <https://doi.org/10.1038/nrn1703>, PMID: 15959466
- Bianchi MT**, Macdonald RL. 2003. Neurosteroids shift partial agonist activation of GABA(A) receptor channels from low- to high-efficacy gating patterns. *The Journal of Neuroscience* **23**:10934–10943. DOI: <https://doi.org/10.1523/JNEUROSCI.23-34-10934.2003>, PMID: 14645489
- Bracamontes JR**, Steinbach JH. 2009. Steroid interaction with a single potentiating site is sufficient to modulate GABA-A receptor function. *Molecular Pharmacology* **75**:973–981. DOI: <https://doi.org/10.1124/mol.108.053629>, PMID: 19176850
- Budelier MM**, Cheng WWL, Bergdoll L, Chen ZW, Janetka JW, Abramson J, Krishnan K, Mydock-McGrane L, Covey DF, Whitelegge JP, Evers AS. 2017. Photoaffinity labeling with cholesterol analogues precisely maps a cholesterol-binding site in voltage-dependent anion channel-1. *Journal of Biological Chemistry* **292**:9294–9304. DOI: <https://doi.org/10.1074/jbc.M116.773069>, PMID: 28396346
- Budelier MM**, Cheng WWL, Chen Z-W, Bracamontes JR, Sugawara Y, Krishnan K, Mydock-McGrane L, Covey DF, Evers AS. 2019. Common binding sites for cholesterol and neurosteroids on a pentameric ligand-gated ion channel. *Biochimica Et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids* **1864**:128–136. DOI: <https://doi.org/10.1016/j.bbalip.2018.11.005>
- Bylund DB**, Deupree JD, Toews ML. 2004. Radioligand-binding methods for membrane preparations and intact cells. *Methods in Molecular Biology* **259**:1–28. DOI: <https://doi.org/10.1385/1-59259-754-8:001>, PMID: 15250483
- Bylund DB**, Toews ML. 1993. Radioligand binding methods: practical guide and tips. *American Journal of Physiology-Lung Cellular and Molecular Physiology* **265**:L421–L429. DOI: <https://doi.org/10.1152/ajplung.1993.265.5.L421>
- Chakrabarti S**, Qian M, Krishnan K, Covey DF, Mennerick S, Akk G. 2016. Comparison of steroid modulation of spontaneous inhibitory postsynaptic currents in cultured hippocampal neurons and Steady-State Single-Channel currents from heterologously expressed $\alpha 1\beta 2\gamma 2$ GABA(A) Receptors. *Molecular Pharmacology* **89**:399–406. DOI: <https://doi.org/10.1124/mol.115.102202>, PMID: 26769414
- Chang Y**, Ghansah E, Chen Y, Ye J, Weiss DS, Chang Y. 2002. Desensitization mechanism of GABA receptors revealed by single oocyte binding and receptor function. *The Journal of Neuroscience* **22**:7982–7990. DOI: <https://doi.org/10.1523/JNEUROSCI.22-18-07982.2002>, PMID: 12223551
- Chen Q**, Wells MM, Arjunan P, Tillman TS, Cohen AE, Xu Y, Tang P. 2018. Structural basis of neurosteroid anesthetic action on GABA_{AA} receptors. *Nature Communications* **9**:3972. DOI: <https://doi.org/10.1038/s41467-018-06361-4>, PMID: 30266951
- Chen ZW**, Bracamontes JR, Budelier MM, Germann AL, Shin DJ, Kathiresan K, Qian MX, Manion B, Cheng WWL, Reichert DE, Akk G, Covey DF, Evers AS. 2019. Multiple functional neurosteroid binding sites on GABA_A receptors. *PLOS Biology* **17**:e3000157. DOI: <https://doi.org/10.1371/journal.pbio.3000157>, PMID: 30845142
- Cheng WWL**, Chen ZW, Bracamontes JR, Budelier MM, Krishnan K, Shin DJ, Wang C, Jiang X, Covey DF, Akk G, Evers AS. 2018. Mapping two neurosteroid-modulatory sites in the prototypic pentameric ligand-gated ion channel GLIC. *Journal of Biological Chemistry* **293**:3013–3027. DOI: <https://doi.org/10.1074/jbc.RA117.000359>, PMID: 29301936

- Comenencia-Ortiz E, Moss SJ, Davies PA. 2014. Phosphorylation of GABAA receptors influences receptor trafficking and neurosteroid actions. *Psychopharmacology* **231**:3453–3465. DOI: <https://doi.org/10.1007/s00213-014-3617-z>, PMID: 24847959
- Das J. 2011. Aliphatic diazirines as photoaffinity probes for proteins: recent developments. *Chemical Reviews* **111**:4405–4417. DOI: <https://doi.org/10.1021/cr1002722>, PMID: 21466226
- Dostalova Z, Zhou X, Liu A, Zhang X, Zhang Y, Desai R, Forman SA, Miller KW. 2014. Human $\alpha 1\beta 3\gamma 2$ l gamma-aminobutyric acid type A receptors: high-level production and purification in a functional state. *Protein Science* **23**:157–166. DOI: <https://doi.org/10.1002/pro.2401>, PMID: 24288268
- Eaton M, Germann A, Arora R, Cao L, Gao X, Shin D, Wu A, Chiara D, Cohen J, Henry Steinbach J, Evers A, Akk G. 2016. Multiple Non-Equivalent interfaces mediate direct activation of GABAA receptors by propofol. *Current Neuropharmacology* **14**:772–780. DOI: <https://doi.org/10.2174/1570159X14666160202121319>
- Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5**:113. DOI: <https://doi.org/10.1186/1471-2105-5-113>, PMID: 15318951
- Evers AS, Chen ZW, Manion BD, Han M, Jiang X, Darbandi-Tonkabon R, Kable T, Bracamontes J, Zorumski CF, Mennerick S, Steinbach JH, Covey DF. 2010. A synthetic 18-norsteroid distinguishes between two neuroactive steroid binding sites on GABAA receptors. *Journal of Pharmacology and Experimental Therapeutics* **333**:404–413. DOI: <https://doi.org/10.1124/jpet.109.164079>, PMID: 20124410
- Farrant M, Nusser Z. 2005. Variations on an inhibitory theme: phasic and tonic activation of GABAA receptors. *Nature Reviews Neuroscience* **6**:215–229. DOI: <https://doi.org/10.1038/nrn1625>
- Feng HJ, Forman SA. 2018. Comparison of $\alpha\beta\delta$ and $\alpha\beta\gamma$ GABA_{AA} receptors: Allosteric modulation and identification of subunit arrangement by site-selective general anesthetics. *Pharmacological Research* **133**:289–300. DOI: <https://doi.org/10.1016/j.phrs.2017.12.031>, PMID: 29294355
- Germann AL, Pierce SR, Burbridge AB, Steinbach JH, Akk G. 2019a. Steady-State activation and modulation of the concatemeric alpha 1 beta 2 gamma 2L GABA(A) Receptor. *Molecular Pharmacology* **96**:320–329. DOI: <https://doi.org/10.1124/mol.119.116913>
- Germann AL, Pierce SR, Senneff TC, Burbridge AB, Steinbach JH, Akk G. 2019b. Steady-state activation and modulation of the synaptic-type $\alpha 1\beta 2\gamma 2$ l GABA_{AA} receptor by combinations of physiological and clinical ligands. *Physiological Reports* **7**:e14230. DOI: <https://doi.org/10.14814/phy2.14230>, PMID: 31549483
- Gielen M, Corringer PJ. 2018. The dual-gate model for pentameric ligand-gated ion channels activation and desensitization. *The Journal of Physiology* **596**:1873–1902. DOI: <https://doi.org/10.1113/JP275100>, PMID: 29484660
- Grobin AC, Gizerian S, Lieberman JA, Morrow AL. 2006. Perinatal allopregnanolone influences prefrontal cortex structure, connectivity and behavior in adult rats. *Neuroscience* **138**:809–819. DOI: <https://doi.org/10.1016/j.neuroscience.2005.12.026>, PMID: 16457952
- Gunduz-Bruce H, Silber C, Kaul I, Rothschild AJ, Riesenberger R, Sankoh AJ, Li H, Lasser R, Zorumski CF, Rubinow DR, Paul SM, Jonas J, Doherty JJ, Kanes SJ. 2019. Trial of SAGE-217 in patients with major depressive disorder. *New England Journal of Medicine* **381**:903–911. DOI: <https://doi.org/10.1056/NEJMoa1815981>, PMID: 31483961
- Haage D, Johansson S. 1999. Neurosteroid modulation of synaptic and GABA-evoked currents in neurons from the rat medial preoptic nucleus. *Journal of Neurophysiology* **82**:143–151. DOI: <https://doi.org/10.1152/jn.1999.82.1.143>, PMID: 10400943
- Harrison NL, Majewska MD, Harrington JW, Barker JL. 1987a. Structure-activity relationships for steroid interaction with the gamma-aminobutyric acidA receptor complex. *The Journal of Pharmacology and Experimental Therapeutics* **241**:346–353. PMID: 3033209
- Harrison NL, Vicini S, Barker JL. 1987b. A steroid anesthetic prolongs inhibitory postsynaptic currents in cultured rat hippocampal neurons. *The Journal of Neuroscience* **7**:604–609. DOI: <https://doi.org/10.1523/JNEUROSCI.07-02-00604.1987>, PMID: 3819824
- Harrison NL, Simmonds MA. 1984. Modulation of the GABA receptor complex by a steroid anaesthetic. *Brain Research* **323**:287–292. DOI: [https://doi.org/10.1016/0006-8993\(84\)90299-3](https://doi.org/10.1016/0006-8993(84)90299-3)
- Hosie AM, Wilkins ME, da Silva HM, Smart TG. 2006. Endogenous neurosteroids regulate GABAA receptors through two discrete transmembrane sites. *Nature* **444**:486–489. DOI: <https://doi.org/10.1038/nature05324>, PMID: 17108970
- Hosie AM, Clarke L, da Silva H, Smart TG. 2009. Conserved site for neurosteroid modulation of GABA A receptors. *Neuropharmacology* **56**:149–154. DOI: <https://doi.org/10.1016/j.neuropharm.2008.07.050>, PMID: 18762201
- Jansen M, Bali M, Akabas MH. 2008. Modular design of Cys-loop ligand-gated ion channels: functional 5-HT3 and GABA rho1 receptors lacking the large cytoplasmic M3M4 loop. *Journal of General Physiology* **131**:137–146. DOI: <https://doi.org/10.1085/jgp.200709896>, PMID: 18227272
- Jayakar SS, Zhou X, Chiara DC, Jarava-Barrera C, Savechenkov PY, Bruzik KS, Tortosa M, Miller KW, Cohen JB. 2019. Identifying drugs that bind selectively to intersubunit general anesthetic sites in the alpha1beta3gamma2 GABAAR transmembrane domain. *Molecular Pharmacology* **95**:615–628. DOI: <https://doi.org/10.1124/mol.118.114975>
- Jayakar SS, Chiara DC, Zhou X, Wu B, Bruzik KS, Miller KW, Cohen JB. 2020. Photoaffinity labeling identifies an intersubunit steroid-binding site in heteromeric GABA type A (GABA_{AA}) receptors. *Journal of Biological Chemistry* **295**:11495–11512. DOI: <https://doi.org/10.1074/jbc.RA120.013452>
- Jiang X, Shu HJ, Krishnan K, Qian M, Taylor AA, Covey DF, Zorumski CF, Mennerick S. 2016. A clickable neurosteroid photolabel reveals selective golgi compartmentalization with preferential impact on proximal

- inhibition. *Neuropharmacology* **108**:193–206. DOI: <https://doi.org/10.1016/j.neuropharm.2016.04.031>, PMID: 27114255
- Jones MV, Westbrook GL. 1995. Desensitized states prolong GABAA channel responses to brief agonist pulses. *Neuron* **15**:181–191. DOI: [https://doi.org/10.1016/0896-6273\(95\)90075-6](https://doi.org/10.1016/0896-6273(95)90075-6), PMID: 7542462
- Jones MV, Westbrook GL. 1996. The impact of receptor desensitization on fast synaptic transmission. *Trends in Neurosciences* **19**:96–101. DOI: [https://doi.org/10.1016/S0166-2236\(96\)80037-3](https://doi.org/10.1016/S0166-2236(96)80037-3), PMID: 9054063
- Kharasch ED, Hollmann MW. 2015. Steroid anesthesia revisited. *Anesthesia & Analgesia* **120**:983–984. DOI: <https://doi.org/10.1213/ANE.0000000000000667>
- Kim JJ, Gharpure A, Teng J, Zhuang Y, Howard RJ, Zhu S, Novello CM, Walsh RM, Lindahl E, Hibbs RE. 2020. Shared structural mechanisms of general anaesthetics and benzodiazepines. *Nature* **585**:303–308. DOI: <https://doi.org/10.1038/s41586-020-2654-5>, PMID: 32879488
- Lange Y, Steck TL. 2016. Active membrane cholesterol as a physiological effector. *Chemistry and Physics of Lipids* **199**:74–93. DOI: <https://doi.org/10.1016/j.chemphyslip.2016.02.003>, PMID: 26874289
- Lavery D, Thomas P, Field M, Andersen OJ, Gold MG, Biggin PC, Gielen M, Smart TG. 2017. Crystal structures of a GABAA-receptor chimera reveal new endogenous neurosteroid-binding sites. *Nature Structural & Molecular Biology* **24**:977–985. DOI: <https://doi.org/10.1038/nsmb.3477>, PMID: 28967882
- Lavery D, Desai R, Uchański T, Masiulis S, Stec WJ, Malinauskas T, Zivanov J, Pardon E, Steyaert J, Miller KW, Aricescu AR. 2019. Cryo-EM structure of the human $\alpha 1\beta 3\gamma 2$ GABAA receptor in a lipid bilayer. *Nature* **565**:516–520. DOI: <https://doi.org/10.1038/s41586-018-0833-4>, PMID: 30602789
- Lee J, Cheng X, Swails JM, Yeom MS, Eastman PK, Lemkul JA, Wei S, Buckner J, Jeong JC, Qi Y, Jo S, Pande VS, Case DA, Brooks CL, MacKerell AD, Klauda JB, Im W. 2016. CHARMM-GUI input generator for NAMD, GROMACS, AMBER, OpenMM, and CHARMM/OpenMM simulations using the CHARMM36 additive force field. *Journal of Chemical Theory and Computation* **12**:405–413. DOI: <https://doi.org/10.1021/acs.jctc.5b00935>, PMID: 26631602
- Li GD, Chiara DC, Sawyer GW, Husain SS, Olsen RW, Cohen JB. 2006. Identification of a GABAA receptor anesthetic binding site at subunit interfaces by photolabeling with an etomidate analog. *The Journal of Neuroscience* **26**:11599–11605. DOI: <https://doi.org/10.1523/JNEUROSCI.3467-06.2006>, PMID: 17093081
- Li P, Shu HJ, Wang C, Mennerick S, Zorumski CF, Covey DF, Steinbach JH, Akk G. 2007. Neurosteroid migration to intracellular compartments reduces steroid concentration in the membrane and diminishes GABA-A receptor potentiation. *The Journal of Physiology* **584**:789–800. DOI: <https://doi.org/10.1113/jphysiol.2007.142794>, PMID: 17761771
- Lorent JH, Levental KR, Ganesan L, Rivera-Longworth G, Sezgin E, Doktorova M, Lyman E, Levental I. 2020. Plasma membranes are asymmetric in lipid Unsaturation, packing and protein shape. *Nature Chemical Biology* **16**:644–652. DOI: <https://doi.org/10.1038/s41589-020-0529-6>, PMID: 32367017
- Lundgren P, Strömberg J, Bäckström T, Wang M. 2003. Allopregnanolone-stimulated GABA-mediated chloride ion flux is inhibited by 3beta-hydroxy-5alpha-pregnan-20-one (isoallopregnanolone). *Brain Research* **982**:45–53. DOI: [https://doi.org/10.1016/S0006-8993\(03\)02939-1](https://doi.org/10.1016/S0006-8993(03)02939-1), PMID: 12915239
- Masiulis S, Desai R, Uchański T, Serna Martin I, Lavery D, Karia D, Malinauskas T, Zivanov J, Pardon E, Kotecha A, Steyaert J, Miller KW, Aricescu AR. 2019. GABAA receptor signalling mechanisms revealed by structural pharmacology. *Nature* **565**:454–459. DOI: <https://doi.org/10.1038/s41586-018-0832-5>, PMID: 30602790
- McGibbon RT, Beauchamp KA, Harrigan MP, Klein C, Swails JM, Hernández CX, Schwantes CR, Wang LP, Lane TJ, Pande VS. 2015. MDTraj: a modern open library for the analysis of molecular dynamics trajectories. *Biophysical Journal* **109**:1528–1532. DOI: <https://doi.org/10.1016/j.bpj.2015.08.015>, PMID: 26488642
- Meng EC, Pettersen EF, Couch GS, Huang CC, Ferrin TE. 2006. Tools for integrated sequence-structure analysis with UCSF chimera. *BMC Bioinformatics* **7**:339. DOI: <https://doi.org/10.1186/1471-2105-7-339>, PMID: 16836757
- Meslamani JE, André F, Petitjean M. 2009. Assessing the geometric diversity of cytochrome P450 ligand conformers by hierarchical clustering with a stop criterion. *Journal of Chemical Information and Modeling* **49**:330–337. DOI: <https://doi.org/10.1021/ci800275k>, PMID: 19434834
- Miller PS, Scott S, Masiulis S, De Colibus L, Pardon E, Steyaert J, Aricescu AR. 2017. Structural basis for GABAA receptor potentiation by neurosteroids. *Nature Structural & Molecular Biology* **24**:986–992. DOI: <https://doi.org/10.1038/nsmb.3484>, PMID: 28991263
- Miller PS, Aricescu AR. 2014. Crystal structure of a human GABAA receptor. *Nature* **512**:270–275. DOI: <https://doi.org/10.1038/nature13293>, PMID: 24909990
- Mitchell EA, Herd MB, Gunn BG, Lambert JJ, Belelli D. 2008. Neurosteroid modulation of GABAA receptors: molecular determinants and significance in health and disease. *Neurochemistry International* **52**:588–595. DOI: <https://doi.org/10.1016/j.neuint.2007.10.007>, PMID: 18055067
- O’Boyle NM, Banck M, James CA, Morley C, Vandermeersch T, Hutchison GR. 2011. Open babel: an open chemical toolbox. *Journal of Cheminformatics* **3**:33. DOI: <https://doi.org/10.1186/1758-2946-3-33>, PMID: 21982300
- Olsen RW. 2018. GABAA receptor: Positive and negative allosteric modulators. *Neuropharmacology* **136**:10–22. DOI: <https://doi.org/10.1016/j.neuropharm.2018.01.036>, PMID: 29407219
- Olsen RW, Sieghart W. 2008. International union of pharmacology LXX subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function update. *Pharmacological Reviews* **60**:243–260. DOI: <https://doi.org/10.1124/pr.108.00505>, PMID: 18790874

- Overstreet LS**, Jones MV, Westbrook GL. 2000. Slow desensitization regulates the availability of synaptic GABA (A) receptors. *The Journal of Neuroscience* **20**:7914–7921. DOI: <https://doi.org/10.1523/JNEUROSCI.20-21-07914.2000>, PMID: 11050111
- Park-Chung M**, Malayev A, Purdy RH, Gibbs TT, Farb DH. 1999. Sulfated and unsulfated steroids modulate gamma-aminobutyric acidA receptor function through distinct sites. *Brain Research* **830**:72–87. DOI: [https://doi.org/10.1016/S0006-8993\(99\)01381-5](https://doi.org/10.1016/S0006-8993(99)01381-5), PMID: 10350561
- Reddy DS**, Estes WA. 2016. Clinical potential of neurosteroids for CNS disorders. *Trends in Pharmacological Sciences* **37**:543–561. DOI: <https://doi.org/10.1016/j.tips.2016.04.003>, PMID: 27156439
- Represa A**, Ben-Ari Y. 2005. Trophic actions of GABA on neuronal development. *Trends in Neurosciences* **28**: 278–283. DOI: <https://doi.org/10.1016/j.tins.2005.03.010>, PMID: 15927682
- Sali A**, Blundell TL. 1993. Comparative protein modelling by satisfaction of spatial restraints. *Journal of Molecular Biology* **234**:779–815. DOI: <https://doi.org/10.1006/jmbi.1993.1626>, PMID: 8254673
- Seljeset S**, Bright DP, Thomas P, Smart TG. 2018. Probing GABA_A receptors with inhibitory neurosteroids. *Neuropharmacology* **136**:23–36. DOI: <https://doi.org/10.1016/j.neuropharm.2018.02.008>, PMID: 29447845
- Shen W**, Mennerick S, Covey DF, Zorumski CF. 2000. Pregnenolone sulfate modulates inhibitory synaptic transmission by enhancing GABA(A) receptor desensitization. *The Journal of Neuroscience* **20**:3571–3579. DOI: <https://doi.org/10.1523/JNEUROSCI.20-10-03571.2000>, PMID: 10804198
- Shen MY**, Sali A. 2006. Statistical potential for assessment and prediction of protein structures. *Protein Science* **15**:2507–2524. DOI: <https://doi.org/10.1110/ps.062416606>, PMID: 17075131
- Sieghart W**. 2015. Allosteric modulation of GABA_A receptors via multiple drug-binding sites. *Advances in Pharmacology* **72**:53–96. DOI: <https://doi.org/10.1016/bs.apha.2014.10.002>, PMID: 25600367
- Sigel E**, Steinmann ME. 2012. Structure, function, and modulation of GABA(A) receptors. *The Journal of Biological Chemistry* **287**:40224–40231. DOI: <https://doi.org/10.1074/jbc.R112.386664>, PMID: 23038269
- Smith SS**, Shen H, Gong QH, Zhou X. 2007. Neurosteroid regulation of GABA(A) receptors: focus on the alpha4 and Delta subunits. *Pharmacology & Therapeutics* **116**:58–76. DOI: <https://doi.org/10.1016/j.pharmthera.2007.03.008>, PMID: 17512983
- Steinbach JH**, Akk G. 2001. Modulation of GABA_A receptor channel gating by pentobarbital. *The Journal of Physiology* **537**:715–733. DOI: <https://doi.org/10.1113/jphysiol.2001.012818>
- Stewart D**, Desai R, Cheng Q, Liu A, Forman SA. 2008. Tryptophan mutations at azi-etomidate photo-incorporation sites on alpha1 or beta2 subunits enhance GABA_A receptor gating and reduce etomidate modulation. *Molecular Pharmacology* **74**:1687–1695. DOI: <https://doi.org/10.1124/mol.108.050500>, PMID: 18805938
- Sugasawa Y**, Bracamontes JR, Krishnan K, Covey DF, Reichert DE, Akk G, Chen Q, Tang P, Evers AS, Cheng WWL. 2019. The molecular determinants of neurosteroid binding in the GABA(A) receptor. *The Journal of Steroid Biochemistry and Molecular Biology* **192**:105383. DOI: <https://doi.org/10.1016/j.jsbmb.2019.105383>, PMID: 31150831
- Tong A**, Petroff JT, Hsu FF, Schmidpeter PA, Nimigeon CM, Sharp L, Brannigan G, Cheng WW. 2019. Direct binding of phosphatidylglycerol at specific sites modulates desensitization of a ligand-gated ion channel. *eLife* **8**:e50766. DOI: <https://doi.org/10.7554/eLife.50766>, PMID: 31724949
- Trott O**, Olson AJ. 2010. AutoDock vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry* **31**:455–461. DOI: <https://doi.org/10.1002/jcc.21334>, PMID: 19499576
- Ueno S**, Zorumski C, Bracamontes J, Steinbach JH. 1996. Endogenous subunits can cause ambiguities in the pharmacology of exogenous gamma-aminobutyric acidA receptors expressed in human embryonic kidney 293 cells. *Molecular Pharmacology* **50**:931–938. PMID: 8863839
- van Meer G**, Voelker DR, Feigenson GW. 2008. Membrane lipids: where they are and how they behave. *Nature Reviews Molecular Cell Biology* **9**:112–124. DOI: <https://doi.org/10.1038/nrm2330>, PMID: 18216768
- Vauquelin G**, Van Liefde I, Swinney DC. 2015. Radioligand binding to intact cells as a tool for extended drug screening in a representative physiological context. *Drug Discovery Today: Technologies* **17**:28–34. DOI: <https://doi.org/10.1016/j.ddtec.2015.09.001>
- Wang M**, He Y, Eisenman LN, Fields C, Zeng CM, Mathews J, Benz A, Fu T, Zorumski E, Steinbach JH, Covey DF, Zorumski CF, Mennerick S. 2002. 3beta -hydroxypregnane steroids are pregnenolone sulfate-like GABA(A) receptor antagonists. *The Journal of Neuroscience* **22**:3366–3375. PMID: 11978813
- Zhu S**, Novello CM, Teng J, Walsh RM, Kim JJ, Hibbs RE. 2018. Structure of a human synaptic GABA_A receptor. *Nature* **559**:67–72. DOI: <https://doi.org/10.1038/s41586-018-0255-3>, PMID: 29950725
- Zhu WJ**, Vicini S. 1997. Neurosteroid prolongs GABA_A channel deactivation by altering kinetics of desensitized states. *The Journal of Neuroscience* **17**:4022–4031. PMID: 9151718
- Ziemba AM**, Szabo A, Pierce DW, Haburcak M, Stern AT, Nourmahad A, Halpin ES, Forman SA. 2018. Alfaxalone binds in inner transmembrane beta+alpha- Interfaces of alpha1beta3gamma2 gamma-Aminobutyric acid type A receptors. *Anesthesiology* **128**:338–351. DOI: <https://doi.org/10.1097/ALN.0000000000001978>
- Zorumski CF**, Paul SM, Covey DF, Mennerick S. 2019. Neurosteroids as novel antidepressants and anxiolytics: gaba-a receptors and beyond. *Neurobiology of Stress* **11**:100196. DOI: <https://doi.org/10.1016/j.yynstr.2019.100196>, PMID: 31649968