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Comparison of Steroid Modulation of Spontaneous Inhibitory Postsynaptic Currents in Cultured Hippocampal Neurons and Steady-State Single-Channel Currents from Heterologously Expressed $\alpha1\beta2\gamma2$L $GABA_A$ Receptors

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ABSTRACT

Neuroactive steroids are efficacious modulators of γ-aminobutyric acid type A receptor ($GABA_A$) receptor function. The effects of steroids on the $GABA_A$ receptor are typically determined by comparing steady-state single-channel open probability or macroscopic peak responses elicited by GABA in the absence and presence of a steroid. Due to differences in activation conditions (exposure duration, concentration of agonist), it is not obvious whether modulation measured using typical experimental protocols can be used to accurately predict the effect of a modulator on native receptors under physiologic conditions. In the present study, we examined the effects of 14 neuroactive steroids and analogs on the properties of spontaneous inhibitory postsynaptic currents (sIPSCs) in cultured rat hippocampal neurons. The goal was to determine whether the magnitude of modulation of the decay time course of sIPSCs correlates with the extent of modulation and kinetic properties of potentiation as determined in previous single-channel studies. The steroids were selected to cover a wide range of efficacy on heterologously expressed rat $\alpha1\beta2\gamma2$L $GABA_A$ receptors, ranging from essentially inert to highly efficacious (strong potentiators of single-channel and macroscopic peak responses). The data indicate a strong correlation between prolongation of the decay time course of sIPSCs and potentiation of single-channel open probability. Furthermore, changes in intracluster closed time distributions were the single best predictor of prolongation of sIPSCs. We infer that the information obtained in steady-state single-channel recordings can be used to forecast modulation of synaptic currents.

Introduction

The γ-aminobutyric acid type A receptor ($GABA_A$) receptor is an inhibitory ionotropic transmitter-gated ion channel whose activation in mature neurons leads to hyperpolarization of the cell or dampening of the effects of excitatory channels. Drugs capable of enhancing $GABA_A$ receptor function have possible applications as anxiolytics, anticonvulsants, and sedatives (Rudolph and Mohler, 2006; Franks, 2008).

Many neuroactive steroids and analogs are potentiators of the mammalian $GABA_A$ receptor. In electrophysiologic experiments, potentiation is observed as augmentation of the whole-cell peak response when a steroid is coapplied with a low concentration of transmitter (Callachan et al., 1987; Harrison et al., 1987a). Studies employing a single-channel patch clamp have revealed that the increase in macroscopic current response is mediated by up to three specific changes in the open and closed time distributions (Akk et al., 2004). Strong potentiators, such as the endogenous steroid (3α,5α)-3-hydroxypregnan-20-one (3α5αP) and the synthetic anesthetic steroid (3α,5α)-3-hydroxypregnane-11,20-dione (3α5αP11O), act by decreasing the prevalence of the long-lived closed state and increasing both the prevalence and mean duration of the

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ABBREVIATIONS: 3α5α16ene17Ph, (3α5α)-17-phenylandrost-16-en-3-ol; 3α5α17βCN, (3α,5α,17β)-3-hydroxyandrostane-17-carbonitrile; 3α5α17βEt, (3α,5α)-pregnan-3-ol; 3α5αP, (3α,5α)-3-hydroxypregnan-20-one; 3α5αP11O, (3α,5α)-3-hydroxypregnane-11,20-dione; 3α5α4βOMe16O, (3α,4β,5α)-3-hydroxyandrostan-17-one; 3α5β17O, (3α,5β)-3-hydroxyandrostan-17-one; 3αCPP17βEp, (2′*S*,3*S*,4a*R*,6a*R*,7a*S*,10a*S*,11a*R*,11b*R*)-hexadecahydro-7a-methyl-spiro[8*H*-cyclopenta[*b*]phenanthrene-8,2′-oxiran]-3-ol; 18nor3α5α17βCN, (3α,5α,17β)-18-nor-3-hydroxyandrostane-17-carbonitrile; *e*3α5β17O, (3β,5α,8α,9β,10α,13α,14β)-3-hydroxyandrostan-17-one; *e*18,19dinor3α5α16O, (3β,5β,8α,9β,10α,13α,14β)-3-hydroxygonan-16-one; *e*19nor3α5α16O, (3β,5β,8α,9β,10α,13α,14β)-3-hydroxyestran-16-one; *e*18nor3α5α16O, (3β,5β,8α,9β,10α,13α,14β)-18-nor-3-hydroxyandrostan-16-one; *e*3α5α4βOMe16O, (3β,4α,5β,8α,9β,10α,13α,14β)-3-hydroxy-4-methoxy-androstan-16-one; *e*3α5α16O, (3β,5β,8α,9β,10α,13α,14β)-3-hydroxyandrostan-16-one; CNQX, 6-cyano-7-nitroquinoxalone-2,3-dione; CT, closed time; DL-APV, DL-2-amino-5-phosphono-valeric acid; DMSO, dimethylsulfoxide; $GABA_A$ receptor, γ-aminobutyric acid type A receptor; OT, open time; P_o, open probability; sIPSC, spontaneous inhibitory postsynaptic current.

long-lived open state (Akk et al., 2005). Weak potentiating steroids, such as (3α,5β)-3-hydroxyandrostan-17-one (3α5β17O), act through changes in a single kinetic component, such as an increase in the prevalence of dwells in the long-lived open state (Li et al., 2007a). There is a good correlation between the magnitude of potentiation of whole-cell peak response and the increase in single-channel open probability (Akk et al., 2010).

Experimental conditions in macroscopic and single-channel studies do not, however, reflect the physiologic conditions in brain. Native $GABA_A$ receptors are continuously bathed in a mixture of steroids of endogenous origin while clearance of exogenously-applied anesthetic steroids occurs slowly, with a timescale of minutes or hours (Ram et al., 2001; Visser et al., 2002). In contrast, drug applications in macroscopic measurements are of finite length, typically lasting a few seconds followed by rapid washout. We have previously found that there is a correlation between the reciprocal of application length and EC_{50} of potentiation (Li et al., 2007b), likely due to initial redistribution of the steroid among the lipid fractions in the cell, that conceals the true extent of drug effect in short applications. Single-channel recordings in the cell-attached configuration are long-lasting, but redistribution of steroid from the patch to the rest of cell, which is acting as a sink, reduces steroid concentration in the patch thereby affecting potency estimates (Li et al., 2007b).

Another shortcoming is the fact that single-channel measurements are obtained under steady-state conditions, but synaptic activity occurs far from the steady state. Native synaptic-type $GABA_A$ receptors are activated by brief (likely $\leq$1 millisecond) pulses of a saturating concentration of transmitter. The distribution of kinetic states occupied during such brief activation may differ from that during prolonged exposure to submaximal agonist concentration in a single-channel recording. Spatial spread and variable rebinding of transmitter are additional potential complications of physiologic, synaptic activation that are not evident in steady-state channel recordings.

The differences in exposure conditions cast some doubt on our ability to accurately predict effects of modulators on native $GABA_A$ receptors under physiologic conditions from the data generated using typical electrophysiologic experimental protocols. In the present study, we have examined the effects of several neuroactive steroids and analogs on the properties of spontaneous inhibitory postsynaptic currents (sIPSCs) in cultured rat hippocampal neurons. The goal was to determine whether the extent of modulation observed in synaptic responses correlates with the kinetic properties of potentiation determined in single-channel studies. On the whole, we infer that the information obtained from steady-state single-channel recordings can be used to forecast modulation of synaptic currents.

Materials and Methods

Cultured rat hippocampal neurons were prepared as described previously elsewhere (Emnett et al., 2015). Rat pups (postnatal day 1–3) were anesthetized with isoflurane, and the hippocampus was dissected and cut into slices (500 μm thickness). The slices were digested with 1 mg/ml papain in oxygenated Leibovitz L-15 medium (Invitrogen, Gaithersburg, MD) followed by mechanical trituration in modified Eagle's medium (Invitrogen) containing 5% horse serum, 5% fetal calf serum, 17 mM D-glucose, 400 μM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were seeded in modified Eagle's medium at a density of ~650 cells mm^{-2} onto 25-mm cover glasses coated with 5 mg/ml of collagen or 0.1 mg/ml of poly-D-lysine with 1 mg/ml laminin. Cultures were incubated at 37°C in a humidified chamber with 5% CO_2/95% air. Cytosine arabinoside (6.7 μM) was added 3 to 4 days after plating to inhibit glial proliferation, followed by replacement of half of the culture medium with Neurobasal medium (Life Technologies, Carlsbad, CA) plus B27 supplement (Life Technologies) the following day. The animal procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health. The protocol was approved by the Animal Studies Committee of Washington University in St. Louis.

We recorded sIPSCs from neurons cultured for 10 to 14 days. For recordings, coverslips with cells were transferred to a new dish with extracellular solution containing (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 D-glucose, and 10 HEPES (pH 7.4 with NaOH). To block glutamate receptors, 5 μM 6-cyano-7-nitroquinoxalone-2,3-dione (CNQX) and 25 μM DL-2-amino-5-phosphono-valeric acid (DL-APV) were added to bath. Steroids and analogs were added to the bath at the indicated concentration at least 10 minutes before recording to reach full equilibration with the drug (Zimmerman et al., 1994). Each coverslip with neurons was exposed to only one kind of drug due to difficulties associated with complete washout of these lipophilic compounds from the cells. The pipette solution contained (in mM): 140 CsCl, 4 NaCl, 4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES (pH 7.4 with CsOH). Neurons were identified visually and clamped at −70 mV. All experiments were done at room temperature.

Currents were amplified with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), low-pass filtered at 1 kHz, and digitized with a Digidata 1322A interface (Molecular Devices) at 5 kHz. The detection and analysis of synaptic currents were conducted using pClamp 10 software (Molecular Devices). First, a template was created by averaging one to three random events under a drug condition. This template was used to identify all the events under that drug condition. The template search parameters were set to detect negative-going peaks of variable amplitude with a template match threshold set at the default value 4, which provides balance between missed events and false positives. The program automatically detected the spontaneous firing events that were then visually inspected and manually accepted or rejected. The overlay plot of all the events thus selected was then saved in a separate file. The events from each recording were averaged, and subsequent analysis was conducted on the averaged traces. Because decay times of the events varied with drug conditions, different templates representing each drug condition had to be created. Decay time courses were fitted to sums of two exponentials. The data are presented in weighted time constants, calculated as $\tau_w = A_1\tau_1 + A_2\tau_2$, where τ_1 and τ_2 are the time constants of the two components and A_1 and A_2 are the proportions of the two components.

Concentration–response curves were fitted for pooled data with the following equation:

$$Y = Y_{min} + (Y_{max}\text{-}Y_{min})*\left([\text{steroid}]^{nH} \Big/ \left([\text{steroid}]^{nH} + EC_{50}^{nH}\right)\right) \quad (1)$$

where EC_{50} is the concentration of steroid producing a half-maximal effect, n_H describes the slope of relationship, and Y_{min} and Y_{max} are the low and high concentration asymptotes, respectively. Fitting was conducted using the NFIT software (University of Texas, Medical Branch at Galveston, Galveston, TX).

Inorganic salts used in the buffers CNQX, and DL-APV were bought from Sigma-Aldrich (St. Louis, MO). Steroids were bought from Sigma-Aldrich or Steraloids (Newport, RI), or synthesized locally as described previously elsewhere (Hu et al., 1993; Scaglione et al., 2008; Li et al., 2009; Qian et al., 2014). Structures of steroids used are given in Fig. 1. Stock solutions of steroids and analogs were made in dimethylsulfoxide (DMSO) at a 10–20 mM concentration. Stock solutions were kept at room temperature and further diluted as

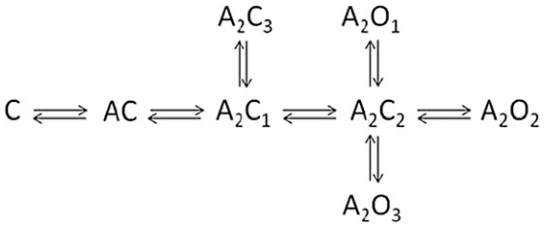
needed on the day of the experiment. The highest final concentration of DMSO was 0.1% (v/v). This concentration of DMSO is without effect on currents from recombinant $\alpha1\beta2\gamma2$L $GABA_A$ receptors and $GABA_A$ receptor-mediated synaptic currents (Li et al., 2007a; Mitchell et al., 2007).

Open probability (P_o) of single-channel activity elicited by 50 μM GABA in the absence or presence of steroids was calculated from the previously published individual intracluster open and closed time distributions using the following equation:

$$P_o = \Sigma\ OT_i * frOT_i / (\Sigma\ OT_i * frOT_i + \Sigma\ CT_i * frCT_i) \quad (2)$$

where OT_i and $frOT_i$ are the mean duration and fraction of the individual open time components and CT_i and $frCT_i$ are the mean duration and fraction of the individual closed time components.

Analysis of single-channel currents and simulation of synaptic events was conducted using the QuB Suite (www.qub.buffalo.edu). Previous single-channel data (Li et al., 2009; Qian et al., 2014) were first reanalyzed using Model 1 (Lema and Auerbach, 2006):



This model predicts three open states, differing in their mean duration, associated with fully liganded receptors. The model and the fitted rate constants were then used to simulate synaptic events. Exposure to a vesicle of GABA was mimicked by an agonist profile with a duration of 1 millisecond and a concentration of 10 mM. The starting state was the unliganded, closed state (C). The decay time courses of resulting macroscopic currents were fitted to a single-exponential, using Origin (OriginLab, Northampton, MA).

Results

Effects of Steroids on the Decay Time Course of sIPSCs. Cells cultured for 10 to 14 days exhibited spontaneous IPSCs in the presence of glutamate receptor blockers CNQX and DL-APV (Fig. 2A). In 12 cells, the mean frequency of events was 0.72 ± 0.69 Hz. The sIPSCs were sensitive to $GABA_A$ receptor blockers and eliminated during bath application of 10 μM gabazine (not shown). The decay time course of averaged sIPSCs was fitted to a sum of two exponentials, yielding the mean weighted time constant (τ_w) of 34 ± 3 milliseconds (mean ± S.E.M.; 12 cells; Fig. 2B). This is similar to several previous estimates for decay times of miniature and spontaneous IPSCs from hippocampal neurons (e.g., Poisbeau et al., 1997; Zorumski et al., 1998; Banks and Pearce, 1999; Park et al., 2011). The amplitudes of sIPSCs varied considerably from cell to cell. There was, however, no correlation between mean amplitude and decay time of sIPSC (Fig. 2B).

Addition of potentiating steroid to the extracellular medium led to an increase in the decay time constant of sIPSCs (Fig. 2A lower trace and Fig. 2C). In the presence of 3 μM 3α5αP11O, the τ_w was 145 ± 16 milliseconds (four cells). The increase in decay time was not accompanied by changes in mean amplitude (149 ± 34 pA versus 175 ± 62 pA under control conditions). Concentration–response measurements conducted in the presence of 10 nM to 3 μM 3α5αP11O yielded an EC_{50} of 0.21 ± 0.11 μM and a Hill coefficient of 1.2 ± 0.8 (data combined from three to five cells at each concentration; Fig. 2D).

The fitted low concentration asymptote (29 milliseconds) was similar to the decay time constant under control conditions. Addition of the endogenous steroid 3α5αP to the extracellular medium also resulted in prolonged sIPSCs. In the presence of 1 μM 3α5αP the τ_w was 145 ± 25 milliseconds (3 cells). The EC_{50} for prolongation of decay time constant was 0.19 ± 0.02 μM. The Hill coefficent was 2.2 ± 0.4, and the low concentration asymptote was at 36 ± 4 milliseconds (Fig. 2D). The maximal fitted values for τ_w in the presence of 3α5αP11O or 3α5αP were indistinguishable (150 ± 22 and 150 ± 7 milliseconds, respectively).

Single-channel experiments have shown that potentiating steroids act on the synaptic-type $\alpha1\beta2\gamma2$L $GABA_A$ receptor via changes in gating properties that manifest as one or more of the following: an increase in the mean duration and prevalence of long openings (duration and % OT3) and a decrease in the prevalence of the closed state associated with channel closing (% CT3). The largest effect on open probability or macroscopic peak response is observed with steroids possessing all three effects (Akk et al., 2010). The steroids 3α5αP and 3α5αP11O modify all three parameters (Akk et al., 2005; unpublished data).

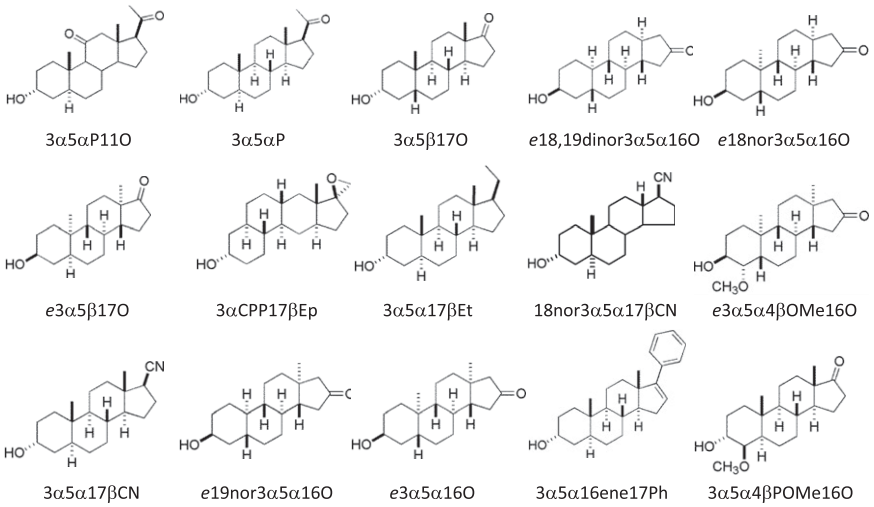


Fig. 1. Structures of steroids and analogs tested in the study. Enantiomeric steroids (*e*18,19dinor3α5α16O, *e*18nor3α5α16O, *e*3α5β17O, *e*3α5α4βOMe16O, *e*19nor3α5α16O, and *e*3α5α16) are inverted relative to natural steroids when bound to $GABA_A$ receptors. For models of the modes of binding natural steroids and their enantiomers, see (Krishnan et al., 2012; Qian et al., 2014).

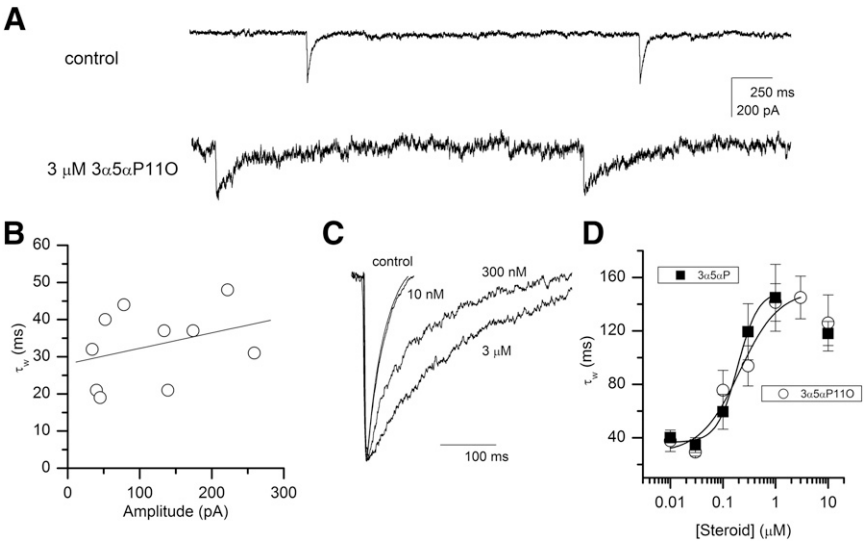


Fig. 2. Properties of sIPSCs. (A) Sample traces showing spontaneous activity under control conditions and in the presence of 3 μM 3α5αP11O. Exposure to the steroid results in prolongation of decay time course and an increase in noise. (B) A relationship between the mean weighted decay time constant and the mean amplitude of sIPSCs under control conditions. An increase in the mean amplitude is not associated with an increase in decay time constant ($R^2 = 0.33, P = 0.35$). (C) Decay time course of sIPSCs under control conditions and in the presence of 10 nM, 300 nM, or 3 μM 3α5αP11O. The traces are averaged from 35 to 297 events per condition and have their amplitudes normalized for better illustration of the effect of steroid on decay time course. (D) Dose-response relationship for steroid-induced prolongation of the weighted decay time constant. The curves were fitted to eq. 1 (*Materials and Methods*). For 3α5αP, $Y_{min} = 36 \pm 4$ milliseconds, $Y_{max} = 150 \pm 7$ milliseconds, $EC_{50} = 0.19 \pm 0.02$ μM, $n_H = 2.2 \pm 0.4$. For 3α5αP11O, $Y_{min} = 29 \pm 5$ milliseconds, $Y_{max} = 150 \pm 22$ milliseconds, $EC_{50} = 0.21 \pm 0.11$ μM, $n_H = 1.2 \pm 0.8$. Exposure to 10 μM 3α5αP or 3α5αP11O resulted in a small reduction of the effect. These data points were not included in the fit.

To probe the relationship between the single-channel mechanism of potentiation and prolongation of τ_w of sIPSCs, we measured spontaneous synaptic activity in the presence of several previously characterized steroids and analogs. Each compound was added to the extracellular solution at a concentration (3 to 10 μM) that was known to produce a saturating response in single-channel or whole-cell peak response measurements.

As expected, steroids that only affect open time distributions in single-channel recordings and have a relatively small effect on macroscopic peak response, had a tendency toward smaller effect on τ_w. In the presence of 10 μM 3α5β17O or (3β,5β,8α,9β,10α,13α,14β)-3-hydroxygonan-16-one [*e*18,19dinor3α5α16O], whose sole effect in single-channel recordings is to increase the relative frequency of long openings (Li et al., 2007a; Qian et al., 2014), the τ_w was 94 $\pm$ 2 milliseconds (5 cells) or 53 $\pm$ 10 milliseconds (four cells), respectively.

We examined the effects of five steroid analogs, (3β,5β,8α,9β,10α,13α,14β)-18-nor-3-hydroxyandrostan-16-one [*e*18nor3α5α16O], (3β,5α,8α,9β,10α,13α,14β)-3-hydroxyandrostan-17-one [*e*3α5β17O], (2′*S*,3*S*,4a*R*,6a*R*,7a*S*,10a*S*,11a*R*,11b*R*)-hexadecahydro-7a-methyl-spiro[8*H*-cyclopenta[*b*]phenanthrene-8,2′-oxiran]-3-ol [3αCPP17βEp], (3α,5α)-pregnan-3-ol [3α5α17βEt], and (3α,5α,17β)-18-nor-3-hydroxyandrostane-17-carbonitrile [18nor3α5α17βCN], that were known to increase both the duration and prevalence of long openings, but not affect intracluster closed times (Li et al., 2007a, 2009; Scaglione et al., 2008; Qian et al., 2014). Exposure to these compounds ranged from no effect on the decay time constant in the presence of *e*18nor3α5α16O ($\tau_w = 36 \pm 2$ milliseconds, four cells) to a more than 4-fold prolongation in the presence of *e*3α5β17O ($\tau_w = 143 \pm 15$ milliseconds, five cells).

In addition to 3α5αP11O and 3α5αP discussed previously, we measured the effects of two additional steroids ((3β,4α,5β,8α,9β,10α,13α,14β)-3-hydroxy-4-methoxy-androstan-16-one [*e*3α5α4βOMe16O] and (3α,5α,17β)-18-nor-3-hydroxyandrostane-17-carbonitrile [3α5α17βCN]) that modify both open and closed times in single-channel recordings producing a strong effect on the peak response (Akk et al., 2004; Qian et al., 2014).

Both compounds also strongly increased the decay time constant of sIPSCs. The τ_w was 243 $\pm$ 24 milliseconds (4 cells) in the presence of *e*3α5α4βOMe16O and 132 $\pm$ 12 milliseconds (4 cells) in the presence of 3α5α17βCN. The steroid (3β,5β,8α,9β,10α,13α,14β)-3-hydroxyestran-16-one [*e*19nor3α5α16O] that only affects closed time properties in single-channel recordings (Qian et al., 2014) was also an efficacious potentiator of the decay time course. In six cells, the decay time constant was 206 $\pm$ 36 milliseconds. We tested the effect of (3β,5β,8α,9β,10α,13α,14β)-3-hydroxyandrostan-16-one [*e*3α5α16O], that in single-channel recordings increases the fraction of long openings and decreases the fraction of long closed times (Qian et al., 2014). The τ_w was 241 $\pm$ 21 milliseconds (6 cells) in the presence of 1 μM *e*3α5α16O.

The steroid (3α5α)-17-phenylandrost-16-en-3-ol (3α5α16ene17Ph) increases the prevalence of OT3 without affecting its mean duration or the closed time distributions. In whole-cell recording, this steroid is essentially without effect on the peak response (Mennerick et al., 2004). Application of 3α5α16ene17Ph weakly increased the decay time of sIPSCs (61 $\pm$ 10 milliseconds; five cells). The data are summarized in Table 1.

As negative control, we measured the effect of (3α,4β,5α)-3-hydroxyandrostan-17-one (3α5α4βOMe16O) on the time course of sIPSCs. Prior macroscopic measurements on heterologously expressed α1β2γ2L receptors had shown that this steroid does not modulate the peak response (Qian et al., 2014). In four cells bathed in 1 μM 3α5α4βOMe16O the τ_w was 33 $\pm$ 4 milliseconds, not different from the value under control conditions ($P > 0.8$; *t* test).

Correlation of Effects on Decay of sIPSCs with Effects on Single-Channel Currents. We next determined whether the magnitude of effects observed on decay time course of sIPSCs correlates with changes in single-channel open and closed time properties in the presence of steroid. For that, we calculated the open probability of the receptor, using eq. 2 (*Materials and Methods*) and previously determined open and closed time parameters (Akk et al., 2004, 2005; Li et al., 2007a; Scaglione et al., 2008; Qian et al., 2014). Results of the calculations and the linear regression fit are shown in Fig. 3A.

TABLE 1

Summary of the effects of steroids on decay time course

The table shows weighted time constants (mean ± S.E.M.) under control conditions (no steroid) and in the presence of saturating concentrations (1–10 μM) of fourteen steroids. The single-channel open probability (P_o) of receptors activated by 50 μM GABA in the absence (control) and presence of steroids is provided for comparison. P_o was calculated from pooled intracluster open and closed time data published previously (Akk et al., 2004, 2005; Li et al., 2007a, 2009; Scaglione et al., 2008; Qian et al., 2014).

Steroid	τ_w	P_o
	ms	
None (control)	34 ± 3	0.41
3α5αP11O	145 ± 16	0.93
3α5αP	145 ± 25	0.84
3α5β17O	94 ± 2	0.44
*e*18,19dinor3α5α16O	53 ± 10	0.46
*e*18nor3α5α16O	36 ± 2	0.49
*e*3α5β17O	143 ± 15	0.72
3αCPP17βEp	86 ± 9	0.83
3α5α17βEt	86 ± 11	0.77
18nor3α5α17βCN	77 ± 16	0.82
*e*3α5α4βOMe16O	243 ± 24	0.86
3α5α17βCN	132 ± 12	0.71
*e*19nor3α5α16O	206 ± 36	0.58
*e*3α5α16O	241 ± 21	0.84
3α5α16ene17Ph	61 ± 10	0.50

The analysis indicates correlation between the increases in τ_w and open probability ($R^2 = 0.56$, $P = 0.029$).

The increase in open probability mainly results from increases in the mean duration and prevalence of the longest-lived open time component, OT3, and a decrease in the prevalence of the longest-lived intracluster closed time component, CT3 (Akk et al., 2004, 2010). To determine whether any single kinetic component correlates with modulation of sIPSCs, we plotted τ_w as a function of changes in each of the three kinetic properties (Fig. 3B–D). The data show no significant correlation with the mean duration of OT3 ($R^2 = 0.19$, $P = 0.50$) or the fraction of OT3 ($R^2 = 0.12$, $P = 0.68$). However, the increase in τ_w showed significant correlation ($R^2 = -0.76$, $P = 0.0001$) with the decrease in the fraction of CT3. We previously (Steinbach and Akk, 2001) assigned this closed state to dwells in the mono- and unliganded closed states. The relative frequency of CT3 indicates how often does the channel return from the numerous diliganded open and closed states to the monoliganded closed state. Thus, steroid modulation of this transition is the best predictor of changes in the decay time constant of sIPSCs.

Modeling of sIPSCs Based on Single-Channel Activation Parameters. We employed Model 1 (*Materials and Methods;* Lema and Auerbach, 2006) to determine whether steroid-induced changes in individual transition rates in single-channel recordings can be used to simulate changes observed in sIPSCs. Modeling was conducted for four conditions: GABA alone, GABA + *e*18,19dinor3α5α16O, GABA + 3α5α17βEt, and GABA + *e*3α5α4βOMe16O. These steroids were selected because they differed in how strongly they modified single-channel responses (Fig. 3). Exposure to *e*18,19dinor3α5α16O results in an increase in the fraction of OT3 (Qian et al., 2014). Both 3α5α17βEt (Li et al., 2009) and *e*3α5α4βOMe16O (Qian et al., 2014) increase both the duration and fraction of OT3, and reduce the fraction of CT3. However, the two steroids differ in the extent of modulation of the fraction of CT3 (Fig. 3D); as a result, the compounds have unequal effects on P_o. The overall rank order of potentiation is: *e*3α5α4βOMe16O > 3α5α17βEt > *e*18,19dinor3α5α16O.

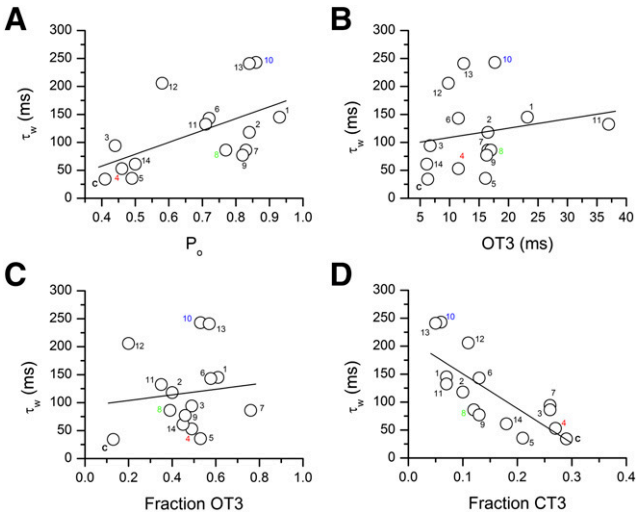


Fig. 3. Correlation between steroid-induced prolongation of sIPSCs and its effect on $\alpha1\beta2\gamma2L$ $GABA_A$ receptor single-channel properties. (A) Steroid-induced increase in τ_w correlates with steroid-induced increase in open probability of single-channel clusters elicited by 50 μM GABA ($R^2 = 0.56$, $P = 0.029$). Each symbol represents data for one condition (control or in the presence of one steroid or analog). C, control (no steroid); 1, 3α5αP11O; 2, 3α5αP; 3, 3α5β17O; 4, *e*18,19dinor3α5α16O; 5, *e*18nor3α5α16O; 6, *e*3α5β17O; 7, 3αCPP17βEp; 8, 3α5α17βEt; 9, 18nor3α5α17βCN; 10, *e*3α5α4βOMe16O; 11, 3α5α17βCN; 12, *e*19nor3α5α16O; 13, *e*3α5α16O; 14, 3α5α16ene17Ph. The structures of steroids are shown in Fig. 1. The data for steroid-effects on single-channel properties are from Akk et al. (2004, 2005); Li et al., (2007a, 2009); Scaglione et al., (2008); and Qian et al., (2014). The data for 3α5α4βOMe16O are not shown. This compound does not enhance the peak macroscopic response; however, its effects on single-channel properties have not been studied. Steroids *e*18,19dinor3α5α16O, 3α5α17βEt, and *e*3α5α4βOMe16O (numbering shown in red, green, and blue, respectively) were used for additional kinetic analysis and modeling summarized in Table 2 and Fig. 4. (B) Steroid-induced increase in τ_w does not correlate with steroid-induced increase in the mean duration of the longest-lived open time component, OT3 ($R^2 = 0.19$, $P = 0.50$). Symbols are coded as in *A*. (C) Steroid-induced increase in τ_w does not correlate with steroid-induced increase in the fraction (relative frequency) of OT3 ($R^2 = 0.12$, $P = 0.68$). Symbols are coded as in *A*. (D) Steroid-induced increase in τ_w correlates with steroid-induced decrease in the fraction of the longest-lived intracluster closed time component, CT3 ($R^2 = -0.76$, $P = 0.0001$). Symbols are coded as in *A*.

For each condition, single-channel data (Li et al., 2009; Qian et al., 2014) from four to six patches were combined and analyzed by fitting to Model 1. Some of the transition rates were fixed to previously determined values. We constrained the GABA association and dissociation rate constants to 3 $\mu M^{-1}s^{-1}$ and 300 $second^{-1}$, respectively (Lema and Auerbach, 2006). Our earlier single-channel data indicate that potentiating steroids do not modify receptor affinity to GABA (Akk et al., 2004). Accordingly, the same values were used in characterization of steroid data. We also constrained the rate constant governing transition from A_2C_1 to A_2C_3 at 300 $second^{-1}$ to improve convergence of fits. The fitted rate constants for all experimental conditions are provided in Table 2.

This analysis provides kinetic correlates to the effects observed in single-channel recordings. All three steroids increase the prevalence of long-lived openings, offset by a decrease in the prevalence of the intermediate-duration open state. This effect is mediated by an increase in the rate of transition from A_2C_2 to A_2O_3, accompanied by a decrease in the rate of transition from A_2C_2 to A_2O_2. Increase in the mean duration of OT3 is mediated by reduction in the $A_2O_3 \rightarrow A_2C_2$ transition rate. The effect on the prevalence of CT3 is mediated

TABLE 2

Results of kinetic modeling

Results of kinetic modeling of single-channel currents elicited by 50 μM GABA alone or in the presence of e18,19dinor3α5α16O, 3α5α17βEt, or e3α5α4βOMe16O. Data from four to six patches at each condition were combined and analyzed using Model 1. The rate constants (and standard deviations estimated from the Hessian matrix) are in s^{-1}. The association (3 $\mu M^{-1}s^{-1}$) and dissociation rate constants (300 s^{-1}) for GABA, and the rate for $A_2C_1 \rightarrow A_2C_3$ transition (300 s^{-1}) were constrained to values determined in a previous analysis (Lema and Auerbach, 2006). Single-channel data were filtered at 2 kHz. The dead time was 90 μs. The rate constants in this table were used to simulate synaptic events (Fig. 4).

Transition	GABA	+e18,19dinor3α5α16O	+3α5α17βEt	+ e3α5α4βOMe16O
$A_2C_1 \rightarrow A_2C_2$	1590 ± 86	1902 ± 103	1972 ± 69	3029 ± 290
$A_2C_2 \rightarrow A_2C_1$	4032 ± 238	3689 ± 287	1846 ± 61	1645 ± 191
$A_2C_3 \rightarrow A_2C_1$	332 ± 28	474 ± 54	586 ± 55	615 ± 67
$A_2C_2 \rightarrow A_2O_1$	1491 ± 116	1185 ± 118	1895 ± 75	1348 ± 79
$A_2O_1 \rightarrow A_2C_2$	4591 ± 317	4648 ± 347	2660 ± 95	2259 ± 120
$A_2C_2 \rightarrow A_2O_2$	3059 ± 254	836 ± 81	920 ± 47	742 ± 80
$A_2O_2 \rightarrow A_2C_2$	404 ± 28	822 ± 107	548 ± 47	206 ± 23
$A_2C_2 \rightarrow A_2O_3$	443 ± 237	1534 ± 106	1401 ± 44	895 ± 86
$A_2O_3 \rightarrow A_2C_2$	138 ± 22	88 ± 3	69 ± 2	47 ± 3

by the rates governing forward and reverse transitions between A_2C_1 and A_2C_2. Interestingly, 3α5α17βEt and e3α5α4βOMe16O, that differ in their maximal effects on the prevalence of CT3, had nonidentical effects on these transitions. Both compounds reduced the rate for $A_2C_2 \rightarrow A_2C_1$, but only e3α5α4βOMe16O increased the rate for $A_2C_1 \rightarrow A_2C_2$ step.

We then simulated synaptic responses using Model 1 and the rate constants in Table 2. The response was driven by a 1-millisecond square-pulse application of 10 mM agonist, with the unliganded, closed state (C) as the starting state. The simulated responses are shown in Fig. 4A along with fits to a single-exponential decay. The predicted decay time constants under the four conditions show the same rank order as measured weighted time constants (GABA + e3α5α4βOMe16O > GABA + 3α5α17βEt > GABA + e18,19dinor3α5α16O > control). The actual values for measured and predicted decay times were within a factor of 3. For receptors activated by GABA alone, we predict that the decay time constant is 18 milliseconds while the average measured τ_w in the absence of steroid was 34 milliseconds. Coapplication of e18,19dinor3α5α16O, 3α5α17βEt, or e3α5α4βOMe16O with GABA prolonged the predicted decay time constant to 37 milliseconds, 66 milliseconds, or 112 milliseconds, respectively. The measured τ_w in the presence of these steroids was 52 milliseconds, 86 milliseconds, and 271 milliseconds. Comparison of predicted and measured responses is shown in Fig. 4B.

Discussion

There are several notable differences between commonly used electrophysiologic recording protocols and the drug exposure conditions in brain. First, exposure times to the modulator are different. In typical experimental protocols, where modulation is determined by comparing responses to an agonist in the absence and presence of a modulator, drug applications normally last from a few seconds in small cells like human embryonic kidney cells or fibroblasts to a few tens of seconds in the case of large cells such as *Xenopus* oocytes. In contrast, the buildup and clearance of many drugs, including lipophilic steroids and analogs, in the brain has a timescale of minutes or hours (Ram et al., 2001; Visser et al., 2002). Our previous work has shown that steroid redistribution to internal lipid compartments affects modulation of cell membrane localized receptors, and that prolonged drug applications result in lower estimated EC_{50} (Li et al., 2007b).

A second issue relates to agonist profile. Both the agonist concentration and application duration are different in the two settings. Experimental modulation is usually measured in the presence of some arbitrary low concentration of agonist, such as EC_5 or EC_{20} GABA for $\alpha1\beta2\gamma2$ receptors, whereas native synaptic $GABA_A$ receptors are alternately bathed in millimolar (saturating) concentrations of GABA after the release of transmitter from presynaptic nerve terminals followed by longer periods where the surrounding medium contains submicromolar ($<EC_1$) concentrations of the agonist. We recently showed that anesthetic drugs readily potentiate the small steady-state currents elicited by submicromolar GABA, intended to mimic ambient GABA between synaptic events (Li and Akk, 2015). It is, however, less clear whether synaptic events can be potentiated, given the high, near-maximal open probability of $\alpha1\beta2\gamma2$ receptors in the presence of saturating GABA. In any case, given the widely different exposure times to the agonist, the occupancy of the various states, and the effects of state-dependent modulators, are likely to be qualitatively different.

We have previously characterized mechanisms of steady-state modulation for several potentiating steroids (e.g., Akk

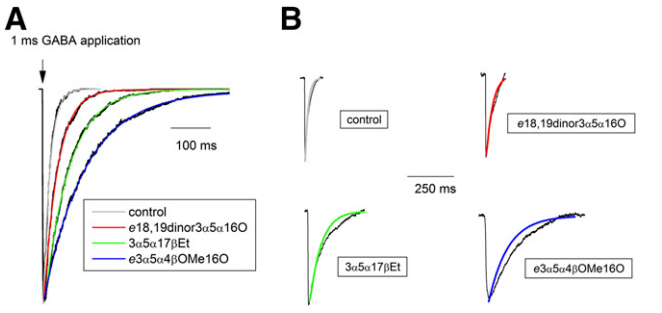


Fig. 4. Modeling of synaptic events. (A) Synaptic events were simulated according to Model 1 and the rate constants given in Table 2. Receptor activation was driven by a 1 millisecond-long square-pulse of 10 mM GABA (arrow) in the absence (control) and presence of saturating concentrations of e18,19dinor3α5α16O, 3α5α17βEt, or e3α5α4βOMe16O. Overlaid on the data traces are single-exponential fits that yielded 18 milliseconds, 37 milliseconds, 66 milliseconds, and 112 milliseconds for control, e18,19dinor3α5α16O, 3α5α17βEt, or e3α5α4βOMe16O, respectively. (B) The fitted lines from (A) are overlaid on the averaged sIPSCs obtained under control conditions (no steroid), and in the presence of e18,19dinor3α5α16O, 3α5α17βEt, or e3α5α4βOMe16O.

et al., 2004). Steroids act by modulating up to three specific parameters of open and closed time distributions. Kinetic components of potentiation detected in single-channel recordings and the resulting changes in receptor open probability are generally a good predictor of magnitude of modulation of peak responses in whole-cell measurements (Li et al., 2007b, 2009; Scaglione et al., 2008; Qian et al., 2014). However, no direct comparison with modulation of synaptic responses is available.

It is known from previous work that addition of neuroactive steroids, such as 3α5αP and 3α5αP11O, to the extracellular medium leads to prolongation of inhibitory postsynaptic currents (Harrison et al., 1987b; Zorumski et al., 1998; Spigelman et al., 2003; Haage et al., 2005). In the present study, we set out to determine whether the magnitude of this effect correlates with the degree of potentiation observed in steady-state single-channel patch clamp recordings, and whether an effect on synaptic currents can be predicted from the kinetic profile of a steroid as determined in single-channel studies.

Based on measuring the effects of fourteen neuroactive steroids and analogs, we conclude that there is a strong positive correlation between steroid-induced changes in single-channel open probability and prolongation of decay time of sIPSCs in the presence of steroids. Steroids that most efficaciously potentiate P_o typically had the strongest effect on τ_{dec}. When we separated the increase in P_o into major components that produce potentiation, we found that the decrease in the prevalence of the longest-lived intracluster closed time component was the sole predictor of prolongation of sIPSCs. This finding is not necessarily surprising because the decrease in the prevalence of CT3 is most strongly associated with enhancement of single-channel P_o and the macroscopic peak response (Akk et al., 2010). In the framework of Model 1, this kinetic effect is jointly produced by the increase in the rate of the $A_2C_1 \rightarrow A_2C_2$ transition and a decrease in the rate of $A_2C_2 \rightarrow A_2C_1$. We interpret the lack of correlation between open time properties and τ_{dec} as an independence of steroid's ability to prolong the mean open duration and its effects on closed times.

Overall, our data indicate that studies of steroid-induced changes in steady-state single-channel currents can be employed to predict steroid effects on transient, synaptic responses. We also infer that the α1β2γ2L receptor is an acceptable model system to mimic and study synaptic-type $GABA_A$ receptors.

Several prior studies have observed prolongation of the decay time course of sIPSCs or deactivation time constant of heterologously expressed α1β2γ2L receptors in the presence of potentiating steroids (Harrison et al., 1987b; Wohlfarth et al., 2002; Spigelman et al., 2003; Haage et al., 2005) or volatile anesthetics such as halothane and isoflurance (Banks and Pearce, 1999). Based on kinetic modeling simulations, Haage et al. (2005) proposed that 3α5αP increases the decay time by reducing the GABA unbinding rate. A similar conclusion was reached for halothane-induced prolongation of decay time course (Li and Pearce, 2000). However, mechanistic conclusions can be dependent on the activation model selected for analysis. Changes in the occupancies of any of the fully liganded states, including various short-lived nonconducting states, would modify the macroscopic deactivation time course (Bianchi and Macdonald, 2001; Bianchi et al., 2007). We previously showed that receptor affinity to GABA, i.e., the binding and unbinding rates, or the maximal effective opening rate in the presence of GABA are not affected by potentiating steroids (Akk et al., 2004). Our current modeling results are in agreement with this, showing that steroid effects can be fully accounted for by changes in transitions between fully liganded states.

Strictly speaking, an increase in the decay time course does not necessarily lead to an increase in charge transfer. Sojourns in intraburst *nonconducting* states have been associated with prolonged decay after brief applications of agonist (Jones and Westbrook, 1995). However, a compound whose sole effect is an introduction of such nonconducting states will also reduce the open probability within the burst. In fact, the prolongation of the burst duration will be exactly offset by the reduction in open probability within the burst. It is noteworthy that the effects observed in the presence of *e*18,19dinor3α5α16O, 3α5α17βEt, or *e*3α5α4βOMe16O increase both the mean duration and the open probability of a burst.

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Authorship Contributions

Participated in research design: Chakrabarti, Covey, Mennerick, Akk.

Conducted experiments: Chakrabarti.

Contributed new reagents or analytic tools: Qian, Krishnan.

Performed data analysis: Chakrabarti, Akk.

Wrote or contributed to the writing of the manuscript: Chakrabarti, Covey, Mennerick, Akk.

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