Comparison of steroid modulation of spontaneous inhibitory postsynaptic currents in cultured hippocampal neurons and steady-state single-channel currents from heterologously expressed α1β2γ2L GABA$_A$ receptors

Sampurna Chakrabarti, Mingxing Qian, Kathiresan Krishnan, Douglas F. Covey, Steven Mennerick, and Gustav Akk

Primary laboratory of origin: Department of Anesthesiology, Washington University School of Medicine, St. Louis, MO, USA

Departments of Anesthesiology (SC, GA), Developmental Biology (MQ, KK, DFC), and Psychiatry (SM), and the Taylor Family Institute for Innovative Psychiatric Research (DFC, SM, GA), Washington University School of Medicine, St. Louis, MO, USA

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Corresponding author:

Gustav Akk, Department of Anesthesiology, Washington University, Campus Box 8054, 660

South Euclid Ave, St. Louis, MO 63110. Tel: (314) 362-3877; Fax: (314) 362-8571; Email:

akk@morpheus.wustl.edu

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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,3S,4aR,6aR,7aS,10aS,11aR,11bR)-

Hexadecahydro-7a-methyl-spiro[8H-cyclopenta[b]phenanthrene-8,2'-oxiran]-3-ol;

18nor3α5α17βCN, (3α,5α,17β)-18-Nor-3-hydroxyandrostane-17-carbonitrile; e3α5β17O,

(3β,5α,8α,9β,10α,13α,14β)-3-Hydroxyandrostan-17-one; e18,19dinor3α5α16O,

(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; GABA$_A$ receptor, γ-aminobutyric acid

type A receptor.

ABSTRACT

Neuroactive steroids are efficacious modulators of 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 physiological conditions. In the present study, we examined the effects of fourteen neuroactive steroids and analogues 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 α1β2γ2L 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 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 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 (Franks, 2008; Rudolph and Mohler, 2006).

Many neuroactive steroids and analogues are potentiators of the mammalian GABA$_A$ receptor. In electrophysiological 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 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 3α5αP and the synthetic anesthetic steroid 3α5αP11O, act by decreasing the prevalence of a long-lived closed state and increasing both the prevalence and mean duration of a long-lived open state (Akk et al., 2005). Weak potentiating steroids, such as 3α5β17O, act through changes in a single kinetic component, e.g., 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 physiological 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

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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, that 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, while synaptic activity occurs far from steady-state. Native synaptic-type $GABA_A$ receptors are activated by brief, likely less or equal to 1 ms, 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 physiological, 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 physiological conditions from the data generated using typical electrophysiological experimental protocols. In the present study, we have examined the effects of several neuroactive steroids and analogues 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 (Emnett et al.,
2015). Rat pups (postnatal day 1-3) were anesthetized with isoflurane, the hippocampus
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 collagen or 0.1 mg/ml 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 3–4 days after plating to inhibit glial proliferation, followed by
replacement of half of the culture medium with Neurobasal medium (Life Technologies) 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.

Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded 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, 10
HEPES (pH 7.4 with NaOH). To block glutamate receptors, 5 µM CNQX and 25 µM DL-APV
were added to bath. Steroids and analogues were added to the bath at the indicated
concentration at least 10 min 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 1-3 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 that 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. Since 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:

$$Y = Y_{min} + (Y_{max} - Y_{min}) * ([steroid]^{nH} / ([steroid]^{nH} + EC_{50}^{nH}))$$
Eq. 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 (The University of Texas, Medical
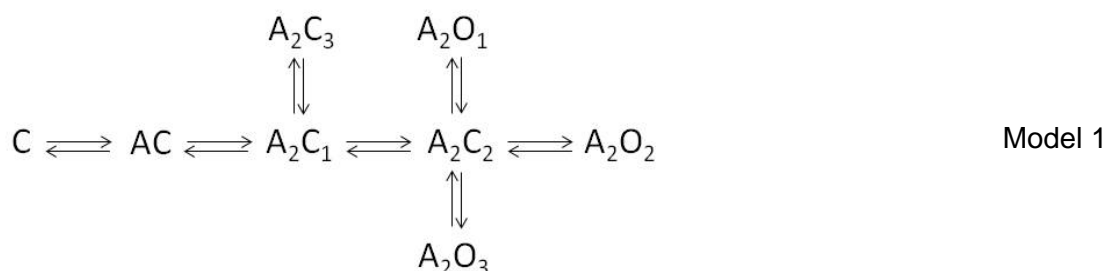
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Branch at Galveston, Galveston, TX).

Inorganic salts used in 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 (Hu et al., 1993; Li et al., 2009; Qian et al., 2014; Scaglione et al., 2008). Structures of steroids used are given in Figure 1. Stock solutions of steroids and analogues were made in DMSO at 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 $\alpha1\beta2\gamma2L$ 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:

$$P_o = \Sigma\ OT_i * frOT_i\ /\ (\Sigma\ OT_i * frOT_i + \Sigma\ CT_i * frCT_i) \qquad \text{Eq. 2}$$

where OT_i, $frOT_i$ are the mean duration and fraction of the individual open time components and CT_i, $frCT_i$ 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 re-analyzed using Model 1 (Lema and Auerbach, 2006):

$$
\begin{array}{ccccc}
 & A_2C_3 & & A_2O_1 & \\
 & \Updownarrow & & \Updownarrow & \\
C \rightleftharpoons AC \rightleftharpoons A_2C_1 & \rightleftharpoons & A_2C_2 & \rightleftharpoons & A_2O_2 \qquad \text{Model 1} \\
 & & \Updownarrow & & \\
 & & A_2O_3 & &
\end{array}
$$

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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 ms 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 Corp., Northampton, MA).

RESULTS

Effects of steroids on the decay time course of sIPSCs

Cells cultured for 10-14 days exhibited spontaneous IPSCs in the presence of glutamate receptor blockers CNQX and DL-APV (Figure 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 ms (mean ± S.E.M.; 12 cells; Figure 2B). This is similar to several previous estimates for decay times of miniature and spontaneous IPSCs from hippocampal neurons (e.g., (Banks and Pearce, 1999; Park et al., 2011; Poisbeau et al., 1997)). The amplitudes of sIPSCs varied considerably from cell to cell. There was, however, no correlation between mean amplitude and decay time of sIPSC (Figure 2B).

Addition of potentiating steroid to the extracellular medium led to an increase in the decay time constant of sIPSCs (Figure 2A lower trace and Figure 2C). In the presence of 3 µM 3α5αP11O, the τ_w was 145 ± 16 ms (4 cells). The increase in decay time was not accompanied by changes in mean amplitude (149 ± 34 pA vs. 175 ± 62 pA under control conditions). Concentration-response measurements conducted in the presence of 10 nM - 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 3-5 cells at each concentration; Figure 2D). The fitted low concentration asymptote (29 ms) 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 ms (3 cells). The EC_{50} for prolongation of decay time constant was 0.19 ± 0.02 µM. The Hill coeffient was 2.2 ± 0.4, and the low concentration asymptote was at 36 ± 4 ms (Figure 2D). The maximal fitted values for τ_w in the presence of 3α5αP11O or 3α5αP

were indistinguishable (150 ± 22 and 150 ± 7 ms, respectively).

Single-channel experiments have shown that potentiating steroids act on the synaptic-type α1β2γ2L 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).

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 analogues. 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 towards smaller effect on τ_w. In the presence of 10 µM 3α5β17O or *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 ± 2 ms (5 cells) or 53 ± 10 ms (4 cells), respectively.

We examined the effects of five steroid analogues, *e*18nor3α5α16O, *e*3α5β17O, 3αCPP17βEp, 3α5α17βEt, and 18nor3α5α17βCN, that were known to increase both the duration and prevalence of long openings, but not affect intracluster closed times (Li et al., 2009; Li et al., 2007a; Qian et al., 2014; Scaglione et al., 2008). Exposure to these compounds ranged from no effect on the decay time constant in the presence of *e*18nor3α5α16O (τ_w = 36 ± 2 ms, 4 cells) to a more than four-fold prolongation in the presence of *e*3α5β17O (τ_w = 143 ± 15 ms, 5 cells).

In addition to 3α5αP11O and 3α5αP discussed above, we measured the effects of two additional steroids (e3α5α4βOMe16O and 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 ± 24 ms (4 cells) in the presence of e3α5α4βOMe16O and 132 ± 12 ms (4 cells) in the presence of 3α5α17βCN. The steroid e19nor3α5α16O 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 ± 36 ms. We tested the effect of e3α5α16O, 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 ± 21 ms (6 cells) in the presence of 1 μM e3α5α16O.

The steroid 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 ± 10 ms; 5 cells). The data are summarized in Table 1.

As negative control, we measured the effect of 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 ± 4 ms, 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 (Methods) and previously determined open and closed time parameters (Akk et al., 2004; Akk et al., 2005; Li et al., 2007a; Qian et al., 2014; Scaglione et al., 2008). Results of the calculations and the linear regression fit are shown in Figure 3A. 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 (Figure 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 (Methods; (Lema and Auerbach, 2006)) to determine whether steroid-induced changes in individual transition rates in single-channel recordings can be used to predict changes observed in sIPSCs. Modeling was conducted for four conditions: GABA alone, GABA + *e*18,19dinor3α5α16O, GABA + 3α5α17βEt, and GABA + *e*3α5α4βOMe16O.

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These steroids were selected because they differed in how strongly they modified single-channel responses (Figure 3). Exposure to e18,19dinor3α5α16O results in an increase in the fraction of OT3 (Qian et al., 2014). 3α5α17βEt (Li et al., 2009) and e3α5α4βOMe16O (Qian et al., 2014) increase both the duration and fraction of OT3, and reduce the fraction of CT3. However, the two steroid differ in the extent of modulation of the fraction of CT3 (Figure 3D); as a result the compounds have unequal effects on P_o. The overall rank order o fpotentiation is: e3α5α4βOMe16O > 3α5α17βEt > e18,19dinor3α5α16O.

For each condition, single-channel data (Li et al., 2009; Qian et al., 2014) from 4-6 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 s^{-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 s^{-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 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 non-identical 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.

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We then simulated synaptic responses using Model 1 and the rate constants in Table 2. The response was driven by a 1 ms square-pulse application of 10 mM agonist, with the unliganded, closed state (C) as the starting state. The simulated responses are shown in Figure 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 three. For receptors activated by GABA alone, we predict that the decay time constant is 18 ms while the average measured τ_w in the absence of steroid was 34 ms. Coapplication of e18,19dinor3α5α16O, 3α5α17βEt, or e3α5α4βOMe16O with GABA prolonged the predicted decay time constant to 37 ms, 66 ms, or 112 ms, respectively. The measured τ_w in the presence of these steroids was 52 ms, 86 ms, and 271 ms. Comparison of predicted and measured responses is shown in Figure 4B.

DISCUSSION

There are several notable differences between commonly-used electrophysiological recording protocols and the drug exposure conditions in brain. First, exposure times to the modulator are different. In typical experimental protocols, modulation is determined by comparing responses to an agonist in the absence and presence of a modulator whereas drug applications normally last from a few seconds in small cells like HEK 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 analogues, 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, e.g., EC_5 or EC_{20} GABA for α1β2γ2 receptors, whereas native synaptic $GABA_A$ receptors are alternately bathed in millimolar (saturating) concentrations of GABA following 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 α1β2γ2 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 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., 2009; Li et al., 2007b; Qian et al., 2014; Scaglione et al., 2008). However, no direct comparison to 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 (Haage et al., 2005; Harrison et al., 1987b; Spigelman et al., 2003; Zorumski et al., 1998). 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 analogues, 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 strongest 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

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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 (Haage et al., 2005; Harrison et al., 1987b; Spigelman et al., 2003; Wohlfarth et al., 2002) or volatile anesthetics such as halothane and isoflurance (Banks and Pearce, 1999). Based on kinetic modeling simulations, Haage and coworkers (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 non-conducting states, would modify the macroscopic deactivation time course (Bianchi et al., 2007; Bianchi and Macdonald, 2001). 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 *non-conducting* states have been associated with prolonged decay following brief applications of agonist (Jones and Westbrook, 1995). However, a compound whose sole effect is an introduction of such non-conducting states will also reduce the open probability within the burst. In fact, the prolongation of the burst duration

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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, and Akk.

Conducted experiments: Chakrabarti.

Contributed new reagents or analytical tools: Qian and Krishnan.

Performed data analysis: Chakrabarti and Akk.

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

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FOOTNOTES

Reprint requests to: Gustav Akk, Department of Anesthesiology, Washington University,

Campus Box 8054, 660 S. Euclid Ave, St. Louis, MO 63110. Email: akk@morpheus.wustl.edu

FIGURE LEGENDS

Figure 1. Structures of steroids and analogues 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).

Figure 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 (Methods). For 3α5αP, Y_{min}

= 36 ± 4 ms, Y_{max} = 150 ± 7 ms, EC_{50} = 0.19 ± 0.02 µM, n_H = 2.2 ± 0.4. For 3α5αP11O, Y_{min} = 29

± 5 ms, Y_{max} = 150 ± 22 ms, EC_{50} = 0.21 ± 0.11 µM, n_H = 1.2 ± 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.

Figure 3. Correlation between steroid-induced prolongation of sIPSCs and its effect on α1β2γ2L

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 analogue). 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 Figure 1. The data for steroid-effects on single-channel properties are from (Akk et al., 2004; Akk et al., 2005; Li et al., 2007a; Li et al., 2009; Qian et al., 2014; Scaglione et al., 2008). 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 Figure 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).

Figure 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 ms-long square-pulse of 10 mM GABA (arrow) in the absence (control) and presence of saturating concentrations of *e*18,19dinor3α5α16O, 3α5α17βEt, or *e*3α5α4βOMe16O. Overlaid on the data traces are single-exponential fits that yielded 18 ms, 37 ms, 66 ms, and 112 ms for control, *e*18,19dinor3α5α16O, 3α5α17βEt, or *e*3α5α4βOMe16O, respectively. (**B**) The fitted lines from

(A) are overlaid on the averaged sIPSCs obtained under control conditions (no steroid), and in

the presence of *e*18,19dinor3α5α16O, 3α5α17βEt, or *e*3α5α4βOMe16O.

Table 1. Summary of the effects of steroids on decay time course.

Steroid	τ_w (ms)	P_o
None (control)	34 ± 3	0.41
3α5αP11O	145 ± 16	0.93
3α5αP	145 ± 25	0.84
3α5β17O	94 ± 2	0.44
e18,19dinor3α5α16O	53 ± 10	0.46
e18nor3α5α16O	36 ± 2	0.49
e3α5β17O	143 ± 15	0.72
3αCPP17βEp	86 ± 9	0.83
3α5α17βEt	86 ± 11	0.77
18nor3α5α17βCN	77 ± 16	0.82
e3α5α4βOMe16O	243 ± 24	0.86
3α5α17βCN	132 ± 12	0.71
e19nor3α5α16O	206 ± 36	0.58
e3α5α16O	241 ± 21	0.84
3α5α16ene17Ph	61 ± 10	0.50

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; Akk et al., 2005; Li et al.,

2007a; Li et al., 2009; Qian et al., 2014; Scaglione et al., 2008).

Table 2. Results of kinetic modeling.

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

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 4-6 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 $µM^{-1}s^{-1}$) 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 (Figure 4).
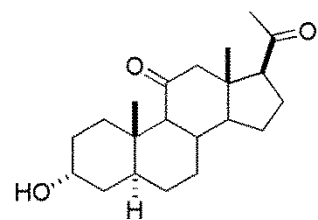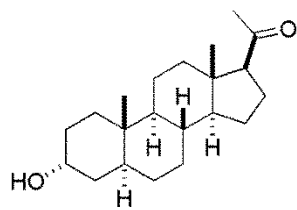
Figure 1
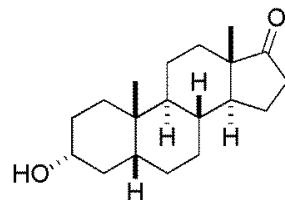
3α5αP11O 3α5αP 3α5β17O *e*18,19dinor3α5α16O *e*18nor3α5α16O
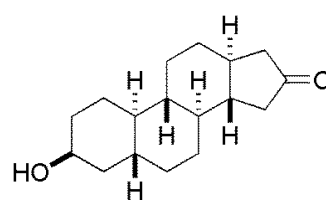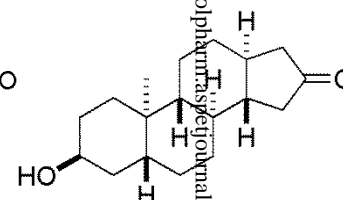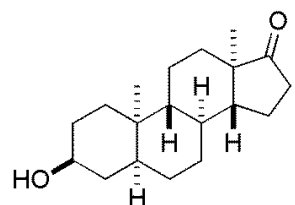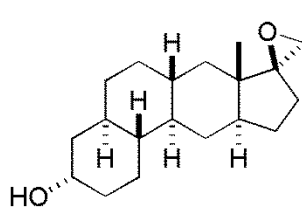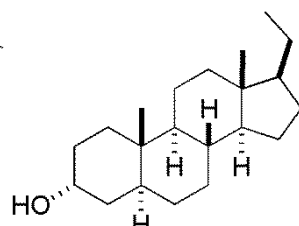
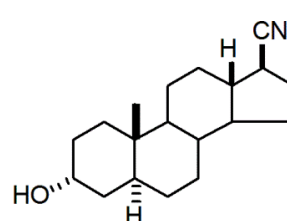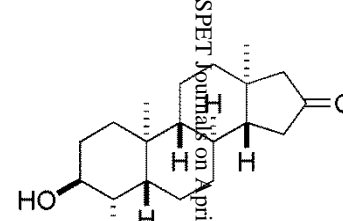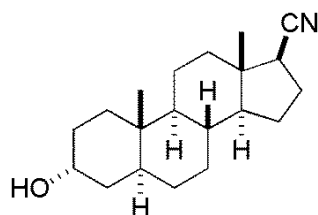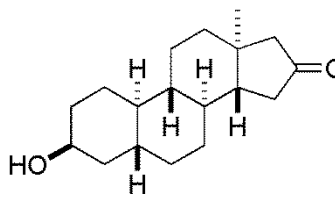*e*3α5β17O 3αCPP17βEp 3α5α17βEt 18nor3α5α17βCN *e*3α5α4βOMe16O
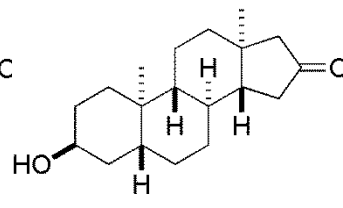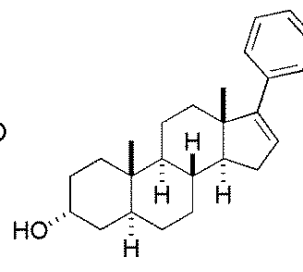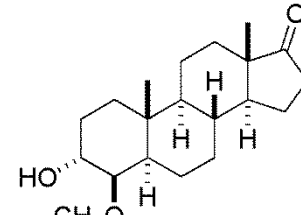
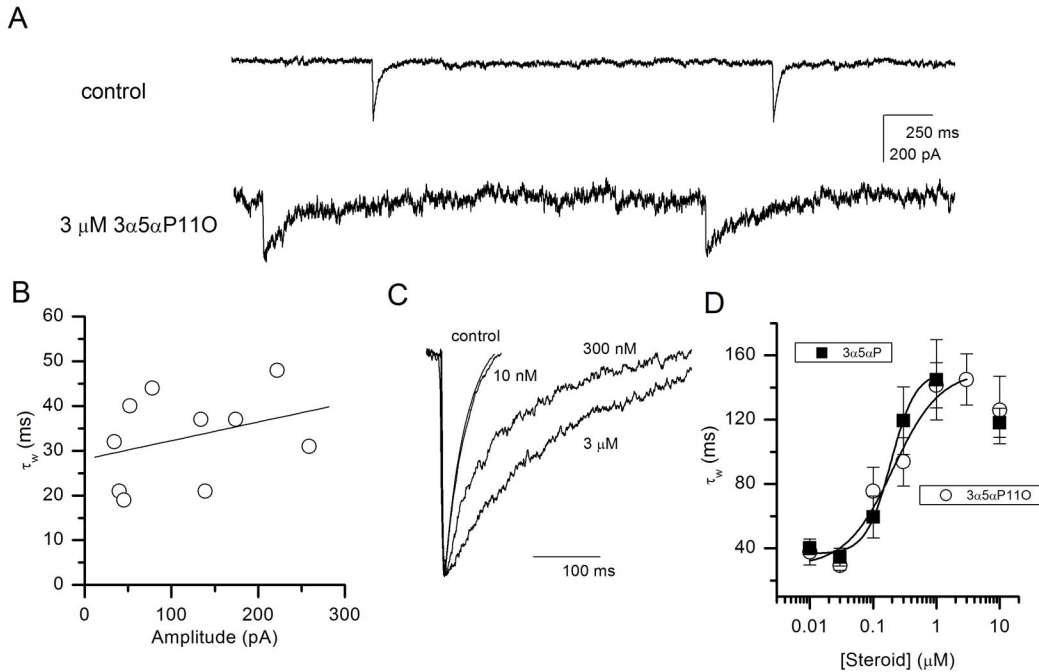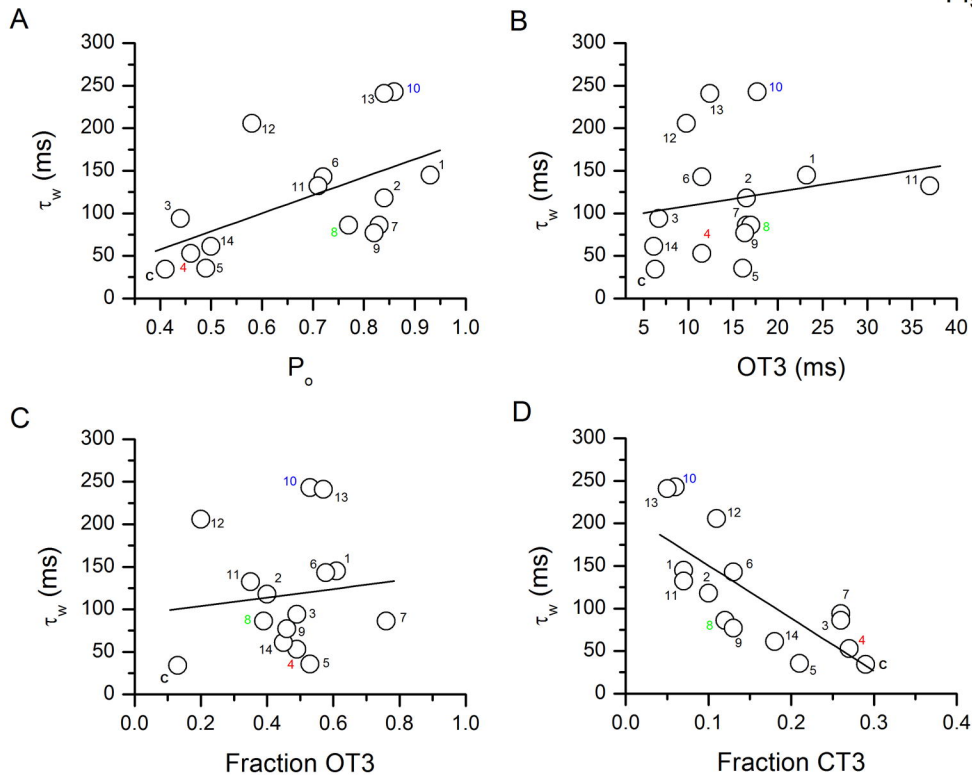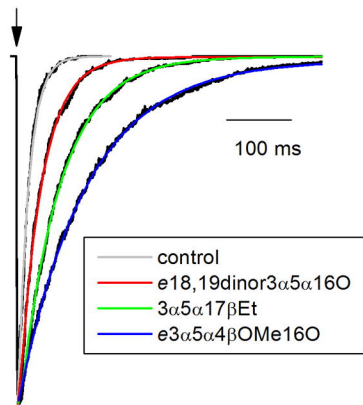3α5α17βCN *e*19nor3α5α16O *e*3α5α16O 3α5α16ene17Ph 3α5α4βPOMe16O

Figure 2

Figure 3

Figure 4

A

1 ms GABA application

100 ms

control
e18,19dinor3α5α16O
3α5α17βEt
e3α5α4βOMe16O

B

control

e18,19dinor3α5α16O

250 ms

3α5α17βEt

e3α5α4βOMe16O