British Journal of Pharmacology

DOI:10.1111/bph.13142
www.brjpharmacol.org

RESEARCH PAPER

Zolpidem and eszopiclone prime α1β2γ2 $GABA_A$ receptors for longer duration of activity

Correspondence
Angelo Keramidas, Queensland Brain Institute, University of Queensland, Brisbane, Qld 4072, Australia. E-mail: a.keramidas@uq.edu.au

Received
17 November 2014
Revised
23 February 2015
Accepted
22 March 2015

Christine L Dixon[1], Neil L Harrison[2], Joseph W Lynch[1,3] and Angelo Keramidas[1,2]

[1]*Queensland Brain Institute, University of Queensland, Brisbane, Qld, Australia,* [2]*Department of Anesthesiology and Department of Pharmacology, Columbia University, New York, NY, USA, and* [3]*School of Biomedical Sciences, University of Queensland, Brisbane, Qld, Australia*

BACKGROUND AND PURPOSE

$GABA_A$ receptors mediate neuronal inhibition in the brain. They are the primary targets for benzodiazepines, which are widely used to treat neurological disorders including anxiety, epilepsy and insomnia. The mechanism by which benzodiazepines enhance $GABA_A$ receptor activity has been extensively studied, but there is little mechanistic information on how non-benzodiazepine drugs that bind to the same site exert their effects. Eszopiclone and zolpidem are two non-benzodiazepine drugs for which no mechanism of action has yet been proposed, despite their clinical importance as sleeping aids. Here we investigate how both drugs enhance the activity of α1β2γ2 $GABA_A$ receptors.

EXPERIMENTAL APPROACH

We used rapid ligand application onto macropatches and single-channel kinetic analysis to assess rates of current deactivation. We also studied synaptic currents in primary neuronal cultures and in heterosynapses, whereby native GABAergic nerve terminals form synapses with HEK293 cells expressing α1β2γ2 $GABA_A$ receptors. Drug binding and modulation was quantified with the aid of an activation mechanism.

KEY RESULTS

At the single-channel level, the drugs prolonged the duration of receptor activation, with similar K_D values of ~80 nM. Channel activation was prolonged primarily by increasing the equilibrium constant between two connected shut states that precede channel opening.

CONCLUSIONS AND IMPLICATIONS

As the derived mechanism successfully simulated the effects of eszopiclone and zolpidem on ensemble currents, we propose it as the definitive mechanism accounting for the effects of both drugs. Importantly, eszopiclone and zolpidem enhanced $GABA_A$ receptor currents via a mechanism that differs from that proposed for benzodiazepines.

Abbreviations

AP-5, D-aminophosphonovaleric acid; CNQX, 7-nitro-2,3-dioxo-1,2,3,4-tetrahydroquinoxaline-6-carbonitrile; TTX, tetrodotoxin

© 2015 The British Pharmacological Society

Tables of Links

TARGETS
Ligand-gated ion channels
GABAA receptors

LIGANDS	
AP-5, D-aminophosphonovaleric acid	GABA
CNQX	TTX, tetrodotoxin
Eszopiclone	Zolpidem

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

$GABA_A$ receptors are selective targets for benzodiazepine-binding site ligands, which have been used clinically for about 50 years to treat a range of neurological disorders including anxiety, epilepsy and insomnia. Heteromeric $GABA_A$ receptors are complexes of five subunits, usually composed of two α (α1–6), two β (β1–3) and a single γ (γ1–3), the most common arrangement being α1β2γ2. Other subunits have also been identified (δ, ε, π and ρ1–3). Each receptor incorporates a central, anion selective permeation pathway (Keramidas *et al.*, 2004). Evidence gleaned from site-directed mutagenesis suggests there is a single high-affinity benzodiazepine-binding site, situated at the interface of α and γ subunits in the extracellular domain of each receptor (Berezhnoy *et al.*, 2004; Rudolph and Knoflach, 2011). This binding site is homologous to the two high-affinity GABA-binding sites found at the interfaces between β and α subunits (Rudolph and Knoflach, 2011).

Mechanistic information drawn from investigations of classical benzodiazepines, such as diazepam, are all consistent with benzodiazepine-induced current enhancement, but vary with regard to the precise stage of the activation process that benzodiazepines perturb. This is partly due to the application of functional schemes that vary in the numbers and connections of discrete functional states (Rogers *et al.*, 1994; Rusch and Forman, 2005; Campo-Soria *et al.*, 2006; Gielen *et al.*, 2012; Li *et al.*, 2013). In contrast to the numerous studies of classical benzodiazepines, there is little mechanistic information for zolpidem and eszopiclone, which are structurally distinct from the classical benzodiazepines, but bind to the benzodiazepine site.

Zolpidem and eszopiclone enhance $GABA_A$ receptor-mediated synaptic currents. Both induce sedation and hypnosis and are clinically important as treatments for sleep disorders (Darcourt *et al.*, 1999; Krystal *et al.*, 2003). Their molecular mechanisms of action have never been investigated in detail. The little information currently available suggests that zolpidem is a more potent modulator than eszopiclone of currents mediated by native α1β2γ2 $GABA_A$ receptors in mouse thalamocortical relay neurons (Jia *et al.*, 2009). However, in recombinant α1β2γ2 $GABA_A$ receptors expressed in *Xenopus laevis* oocytes, estimates of the apparent affinities of the two drugs were similar, at 50–60 nM (Hanson *et al.*, 2008). Zolpidem is reported to be highly selective for α1-containing $GABA_A$ receptors (Korpi *et al.*, 2002; Wingrove *et al.*, 2002), whereas the selectivity of eszopiclone remains uncertain. Finally, site-directed mutagenesis studies of residues in and around the benzodiazepine-binding pocket of α1β2γ2 $GABA_A$ receptors suggest the two modulators may bind in different orientations (Hanson *et al.*, 2008) and enhance GABA-mediated currents via structurally different mechanisms (Morlock and Czajkowski, 2011). The aim of the present study is to investigate $GABA_A$ receptor-mediated current modulation by eszopiclone and zolpidem within the framework of a data-derived activation mechanism that has previously been validated by us and others (Lema and Auerbach, 2006; Dixon *et al.*, 2014), with the addition of an explicit binding step for the modulator.

Methods

Cell culture and transfections

HEK293 cells were transfected with the cDNAs encoding the human α1 (pCIS2), β2 (pcDNA3.1+) and γ2S (pcDNA3.1+) subunits at a plasmid transfection ratio of 1α1 : 1β2 : 3γ2S, using a Ca^{2+} phosphate-DNA co-precipitation method. CD4 or GFP were included as identifiers of transfected cells. To promote the formation of (co-culture) synapses between transfected HEK293 cells and primary rat cortical neurons, neuroligin 2A was also transfected.

Electrophysiology

All recordings were performed at room temperature (22 ± 1°C) at a clamped potential of –70 mV, in whole-cell (synapses) or outside-out patch (single channel and macropatch) patch-clamp configurations. The intracellular solution was composed of (in mM): 145 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 HEPES and 10 EGTA, adjusted to pH 7.4 with CsOH. The extracellular solution was composed of (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 D-glucose, adjusted to pH 7.4 with NaOH.

Single-channel and macropatch currents were recorded using an Axon 200B amplifier (Molecular Devices, Sunnyvale, CA, USA), filtered (–3 dB, 4-pole Bessel) at 5 kHz and sampled at 50 kHz. Synaptic currents were recorded using an Axon 700B amplifier, filtered at 4 kHz and sampled at 10 kHz. Currents were filtered offline to 5 kHz for making figures.

Ensemble current simulations were carried out with QuB software using the schemes shown in Fig. 7 and correspond-

ing rate constants from Table 1. The receptor number for the simulations was set to 1000 and the ligand application time was set to 1 ms (Lema and Auerbach, 2006).

Data analysis

Macropatch and synaptic currents were analysed using pClamp10 (Molecular Devices). 10–20 macropatch currents were elicited from the same patch and averaged for measurement of rise and decay times. The rise and decay times from all patches were then averaged. Similarly, synaptic currents from each cell were selected, averaged for that cell and then combined with data from other cells to obtain total averages. Data represent group means ± SEMs. Group means were tested for significance using one-way ANOVA and pair-wise comparisons were determined using Tukey's test, where $P < 0.05$ was taken as the significance threshold. For further detail on the analysis methods, please refer to Supporting Information.

Materials

GABA and zolpidem were supplied by Sigma-Aldrich (St Louis, MO USA) and eszopiclone by Sepracor/Sunovion ((Marlborough, MA, USA). CNQX and AP-5 were from Tocris (Bristol, UK) and TTX, from Abcam (Cambridge, UK).

Results

Ensemble currents

Two experimental approaches were employed to mimic the effects of 1 μM eszopiclone and zolpidem under synaptic conditions. The first set of experiments involved exposing excised outside-out patches to brief pulses of saturating GABA (±1 μM eszopiclone or zolpidem). The second involved quantitating the effect of both drugs on spontaneous and miniature IPSCs in HEK293-neuronal co-cultures and primary neuronal cultures respectively. Three parameters were measured in these experiments: the peak current amplitude and the current activation and deactivation rates.

Macropatches

The effects of eszopiclone and zolpidem on macropatch currents were examined under two experimental conditions, which differed with respect to drug exposure time. In the first experiment, eszopiclone or zolpidem were co-applied with 5 mM GABA for ≤1 ms to naïve patches (Figure 1A), whereas in the second experiment, the patches were continuously perfused with drug for 1–2 min, prior to co-application with 5 mM GABA plus drug for ≤1 ms (Figure 1B). The aim of these two experimental paradigms was to test if receptors could accumulate in modulator-bound states and thus affect currents.

Current decay was quantified by fitting this phase of the current to two exponential components, then calculating the weighted time constant (Supporting Information). Co-applying drug with GABA produced a slowing of current decay, with higher values of weighted time constants, compared to those for 5 mM GABA alone. A one-way ANOVA indicated that both drugs had a significant effect on current decay ($P < 0.05$), especially zolpidem ($P < 0.01$, Figure 1A, C). A similar effect was observed when the patches were pre-incubated with the drugs for 1–2 min. Again, a one-way ANOVA indicated that both drugs significantly slowed current decay ($P < 0.05$), with zolpidem having the greater effect ($P < 0.01$, Figure 1A, C).

An analysis of the activation rate (see Supporting Information) produced sub-millisecond activation time constants (Figure 1D) and ANOVA showed that only zolpidem accelerated activation ($P = 0.044$). A near identical pattern was observed for current activation when the recorded patches were first pre-incubated with either drug. Again, a significant increase in activation was only observed for zolpidem ($P = 0.030$; Figure 1D).

The rapid, brief application of 5 mM GABA produced peak currents that ranged from 40 to 400 pA (mean, 148 ± 32 pA, $n = 8$). Co-applying or pre-incubating the patches with eszopiclone or zolpidem did not induce any significant change in peak amplitude (Figure 1E). This is in accord with a report showing that zolpidem did not increase peak amplitude in rapid drug application onto macropatches when using saturating concentrations of GABA (Perrais and Ropert, 1999).

IPSCs in HEK293 cell co-cultures and primary neuronal cultures

As eszopiclone and zolpidem affected macropatch currents mainly by slowing the decay rate, we wished to see if these drugs had comparable effects on α1β2γ2 $GABA_A$ receptors expressed in a more realistic synaptic context. As illustrated by the recording in Figure 2A, the effects of both drugs on spontaneous IPSCs (sIPSCs) mediated by recombinant α1β2γ2 $GABA_A$ receptors in HEK293 cells were observed within less than 10 s and were readily reversible. Upon perfusing the recorded cell with either modulator, a net increase in amplitude was observed (Figure 2A, B).

These sIPSCs activated and decayed at rates that were similar to those of macropatch currents. The summary data for these experiments showed that in the sustained presence of eszopiclone or zolpidem, the weighted deactivation time constants were increased compared with those for spontaneous (drug free) currents (Figure 2C, D).

sIPSCs activated ~2-fold more slowly than the macropatch currents, which may indicate that the concentration of GABA is lower at synapses than the 5 mM GABA that was used for the macropatch experiments. However, neither 1 μM eszopiclone nor zolpidem affected the activation time (Figure 2E).

Unlike the negligible increase seen in macropatch recordings, both modulators clearly affected the mean peak current. sIPSC peaks in the absence of drug ranged between 60 and 600 pA (mean, 166 ± 37 pA, $n = 7$) and both drugs increased peak current ($P < 0.05$), especially zolpidem ($P < 0.01$; Figure 2F).

To check whether the amplitude increase was due to enhanced neurotransmitter release, we tested the effects of both drugs on miniature IPSCs (mIPSCs). As mIPSCs could not be recorded with sufficiently high frequency from the heterosynapses, we recorded them from the cultured cortical neurons used in the heterosynapses (Figure 3A, B). Control mIPSCs exhibited a mean decay time constant of 56.1 ± 2.6 ms ($n = 4$), which was greater than in the heterosynapses, possibly reflecting a more complex subunit expression in

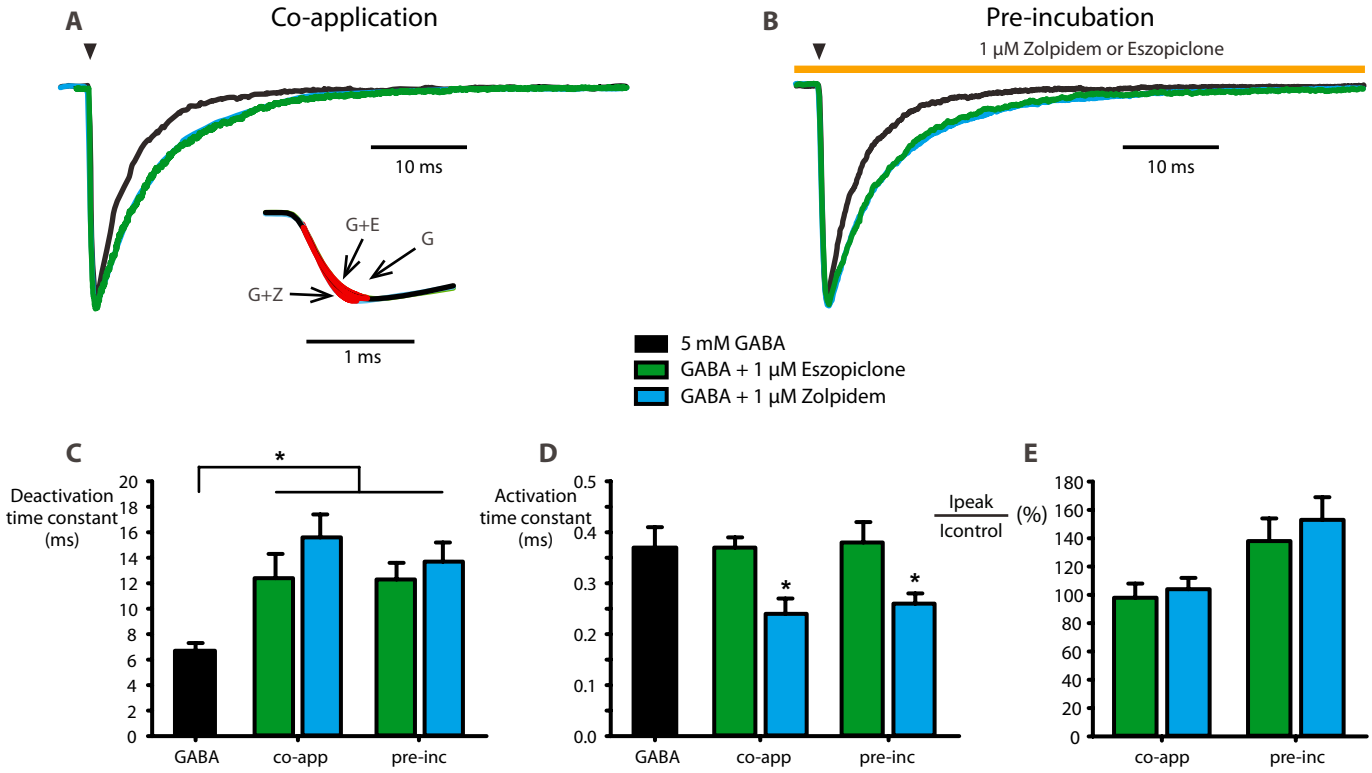

Figure 1

Macropatch currents mediated by α1β2γ2S $GABA_A$ receptors in response to GABA, eszopiclone and zolpidem. (A) Sample recordings of macropatch currents in response to ≤1 ms (arrow head) application of 5 mM GABA or co-application of 5 mM GABA and 1 μM eszopiclone or zolpidem. An expanded view of the activation phase of the currents, along with the 10–100% fits (red) is shown as an inset. (B) Macropatch recordings in response to ≤1 ms (arrow head) application of 5 mM GABA or co-application of 5 mM GABA and 1 μM eszopiclone or zolpidem after 1–2 min pre-incubation of the test drug. Note the similar slowing of current decay for both drugs in A and B. Each current trace in A and B represents an average of 10–20 recordings from the same patch. (C) Group averages of current deactivation rates showing a significant slowing, compared with GABA alone (n = 9), after co-application of 1 μM eszopiclone ($n = 9$) or zolpidem ($n = 5$). Pre-incubation with 1 μM eszopiclone ($n = 8$) or zolpidem ($n = 12$) had similar effects. *$P < 0.05$, significantly different as indicated. (D) Group averages of the activation rates of macropatch currents in the presence of 1 μM eszopiclone ($n = 9$) and zolpidem ($n = 5$). A significant change in activation rate was observed only with zolpidem**.** compared with GABA alone (n = 9). *$P < 0.05$, significantly different from GABA alone. (E) Averaged data of the change in peak current upon exposure to 1 μM eszopiclone and zolpidem. Neither drug produced any significant change in peak current. Currents were recorded at a holding potential of −70 mV.

neurons. However, both eszopiclone and zolpidem produced the expected increase in decay time constant ($P < 0.05$; Figure 3C, D). mIPSCs from neurons had 10–100% activation time constants similar to those in the heterosynapses and activation was not changed by eszopiclone or zolpidem (Figure 3E). As predicted by our previous experiments, both drugs increased amplitude to about the same extent ($P < 0.05$; Figure3F).

As benzodiazepine-binding site ligands increase peak current elicited by sub-saturating concentrations of GABA (Morlock and Czajkowski, 2011; Li *et al.*, 2013), the most likely explanation for the observed increase in peak current is that the net release of GABA at synapses is sub-saturating, as has been postulated for some cortical GABAergic synapses (Perrais and Ropert, 1999). Our ensemble current data suggest that the effects of both drugs are similar and occur upon rapid, brief exposure to the receptors. As exposure times longer than ~1 ms had no additional effect (Figure 1A, B), our data provide no evidence for the accumulation of drug-bound receptors.

Single-channel currents

Open and shut dwell times and open-state occupancy (P_o). The major effect of eszopiclone and zolpidem on macropatch currents was to decrease the decay rate without greatly affecting the activation rate or peak current. We next sought to investigate the mechanism that underlies the increased decay time via single-channel kinetic analysis. Two key parameters of single-channel currents were considered: the effect of drug on the duration of an activation, and the effect of drug on the relative time spent in open states within an activation (P_o). Two types of experiment were carried out to measure these parameters. In the first, the concentration of drug was kept constant at 1 μM while varying the concentration of GABA, whereas in the second, the GABA concentration was maintained at 25 μM while the concentration of drug that was co-applied with GABA was either 5 or 20 μM. In addition to these measurements, we monitored the open and shut dwell components obtained from fits to dwell histograms in the presence of GABA and drugs (Supporting Information).

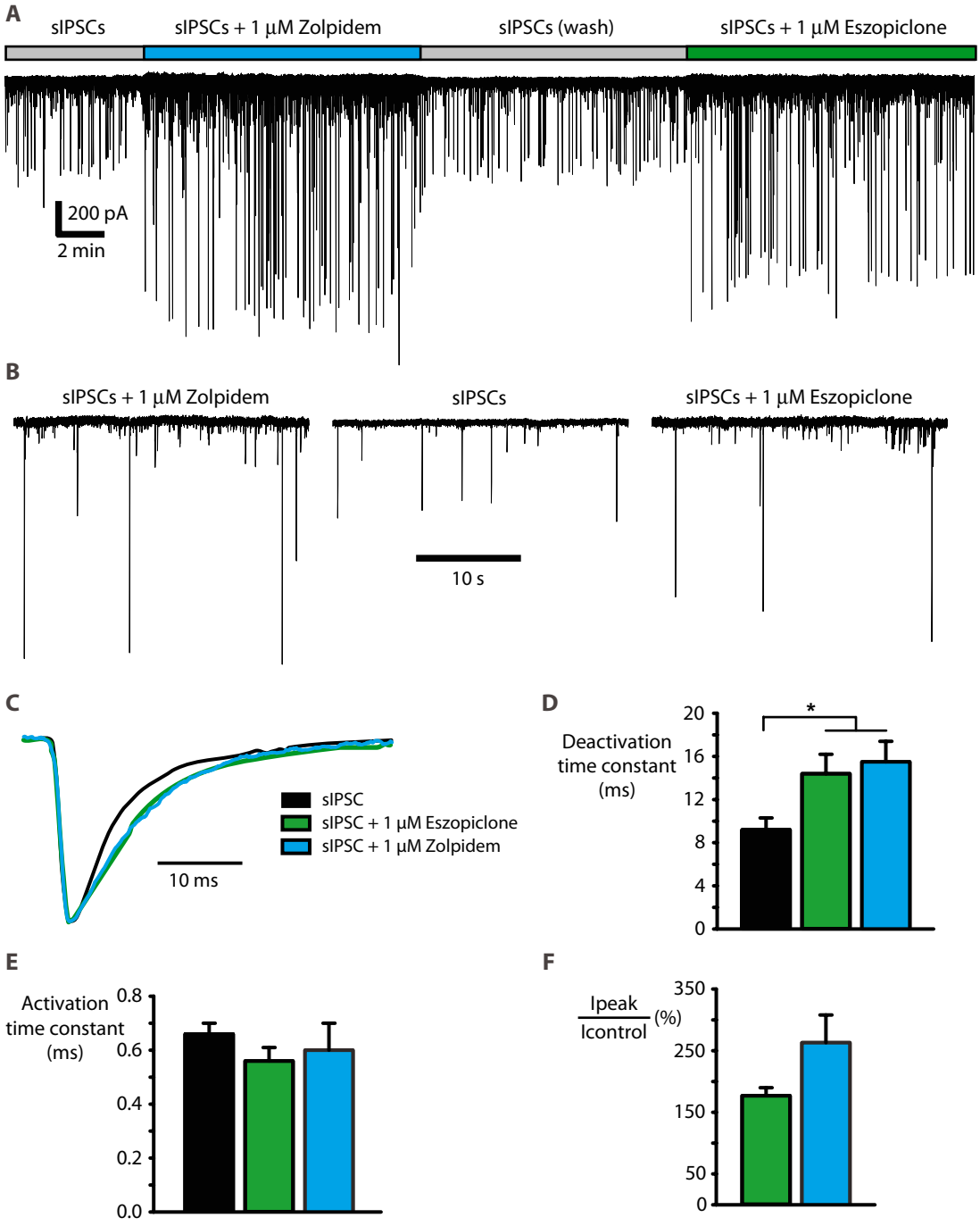

Figure 2

sIPSCs in heterosynapses between neurons and HEK293 cells expressing $\alpha1\beta2\gamma2S$ $GABA_A$ receptors. (A) A whole-cell recording of sIPSCs in the presence and absence of 1 μM eszopiclone or zolpidem. The effects of both drugs were completely reversed within seconds of drug washout. (B) Expanded view of segments of record in A showing that both modulators increased mean peak amplitude. (C) Averaged (typically from 10–20 individual sIPSCs), normalized synaptic currents in the absence of drug or presence of 1 μM eszopiclone or zolpidem showing that both drugs decreased the decay rate of the synaptic currents. (D) Summary data for the deactivation rate of synaptic currents showing that both eszopiclone ($n = 5$) and zolpidem ($n = 5$) slowed this phase of the current, compared with drug-free conditions ($n = 7$). *$P < 0.05$, significantly different as indicated. (E) Averaged data of the activation rate of synaptic currents showing that neither eszopiclone ($n = 5$) nor zolpidem ($n = 5$) had any effect on the drug-free current activation ($n = 7$). (F) Averaged, normalized peak synaptic currents showing that both drugs enhance current amplitude. *$P < 0.05$, significant effect of drug.

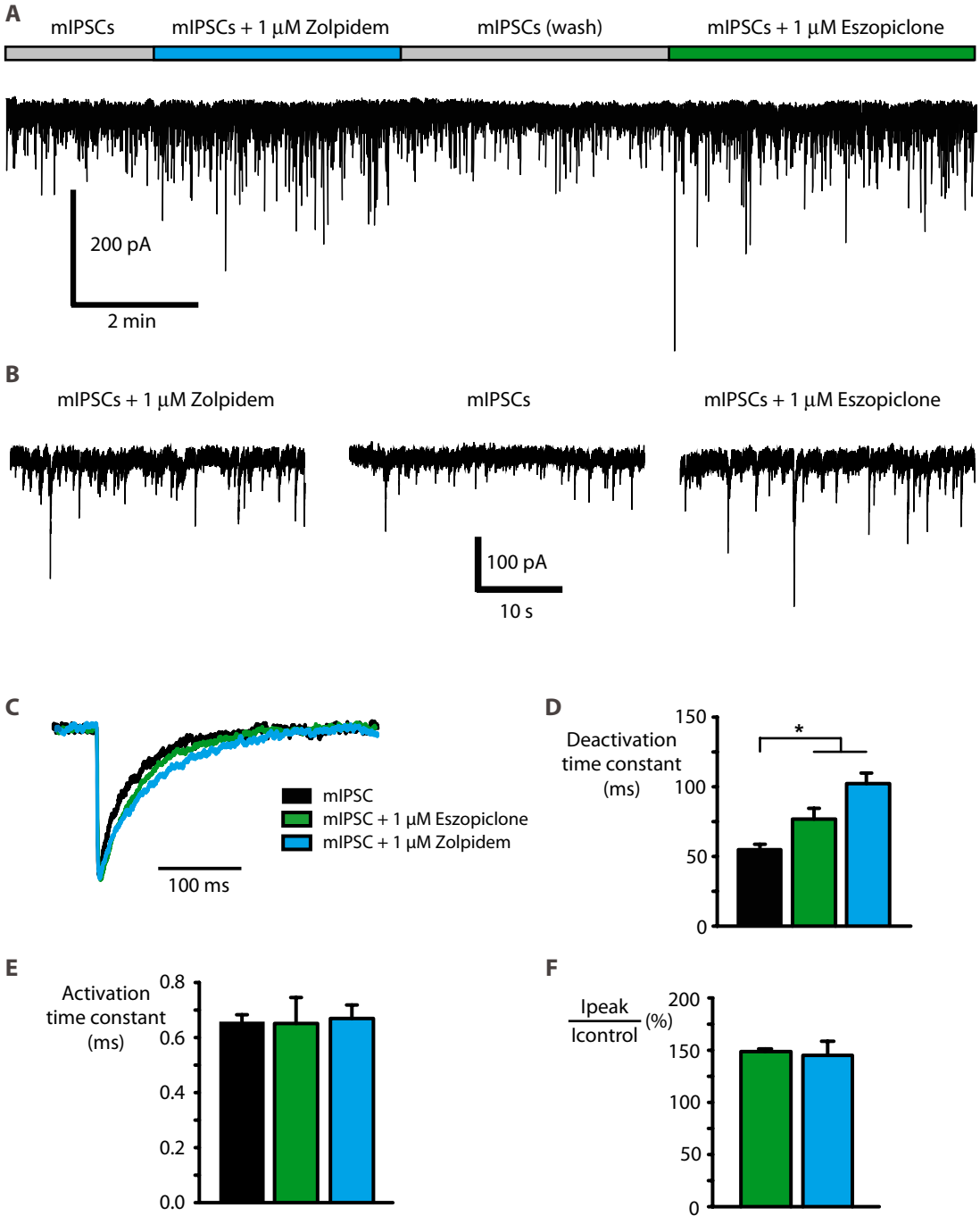

Figure 3

mIPSCs in primary neuronal cultures. (A) Neuronal mIPSCs in the presence and absence of 1 μM eszopiclone or zolpidem. To isolate mIPSCs, the experiments were conducted in the presence of TTX (200 nM), CNQX (10 μM) and AP5 (50 μM). (B) Expanded view of segments of record in A. (C) Averaged and normalized mIPSCs in the absence of drug or presence of 1 μM eszopiclone or zolpidem. (D) Averaged data for the deactivation rate of mIPSCs showing that both eszopiclone ($n = 4$) and zolpidem ($n = 4$) slowed this phase of the current, compmared with the control values ($n = 4$). *$P < 0.05$, significantly different as indicated. (E) Averaged data of the activation rate of synaptic currents showing that activation was unaffected by either drug. (F) Averaged, normalized peak synaptic currents showing that both drugs enhance current amplitude. *$P < 0.05$).

Examples of single-channel currents elicited by increasing GABA concentrations are shown in Figure 4A. Preserving the three briefest shut components resulted in open dwell histograms with either three open (56% of patches) or two open (44% of patches) components. The presence of three shut components that are broadly similar over the concentration range (Figure 4B) suggest that the that the majority of the activations are due to fully (di-) liganded receptors, even at 2 μM GABA, consistent with other data (Macdonald *et al.*, 1989; Lema and Auerbach, 2006; Keramidas and Harrison, 2010). At 5 mM GABA the fraction of the longest shut component decreased by over 9% compared with the mean of the other three concentrations (Figure 4B). The proportion of the longest open component increased by twofold as a function of GABA concentration (Figure 4C). These small changes in dwell components, acting together are consistent with the modest increase in P_o as a function of GABA concentration (Figure 4D). Studies on glycine (Burzomato *et al.*, 2004; Lape *et al.*, 2008) and ACh (Lape *et al.*, 2008) receptors also show dwell components that vary little (or very subtly as ours do) with agonist concentration and give rise to a sigmoidal P_o-[agonist] trend. As illustrated by the group P_o plots, a 1 μM concentration of either drug failed to have any effect on P_o when co-applied with higher concentrations of GABA, although a moderate but significant increase in P_o was seen at 2 μM GABA (Figure 4D). A modest, progressive increase in the P_o was also observed as eszopiclone and zolpidem were increased from 5 to 20 μM, with zolpidem having a more prominent effect (Figure 4E). Overall, the increase in P_o was relatively small and similar for both modulators.

Mean durations of single-channel activations. A greater effect of eszopiclone and zolpidem was seen in the duration of activation. Examples of these recordings for two GABA concentrations and 1 μM modulator are shown in Figure 5A and the group data are shown plotted as a function of GABA concentration in Figure 5B. At 2 μM GABA, 1 μM eszopiclone and zolpidem increased the mean activation duration by about 1.7-fold. Mean activation durations at 5 mM GABA were similarly increased by 1.3 –fold when 1 μM eszopiclone or zolpidem was co-applied. When 1 μM drug was co-applied with either 5 mM, 200 μM or 2 μM GABA the shut and open dwell components varied from those in GABA alone, with the same trend apparent for both drugs. The shortest shut components remained constant, whereas the second and third decreased and increased respectively (Figure 5C). There was an increase in all three open dwell time constants in the presence of either drug and the fractions of the first and second become nearly equal (Figure 5D).

Fixing the GABA concentration at 25 μM and varying the concentration of eszopiclone or zolpidem to 5 or 20 μM (Figure 6A) also produced an increase in the durations of active periods per receptor. However, the increase at 5 μM drug, though significant, was the same as that at 20 μM, for either eszopiclone or zolpidem (Figure 6B). Furthermore, ANOVA performed on the drug-treated groups indicated no significant difference in mean activation durations between drugs at both 5 and 20 μM concentrations. These data indicate that the eszopiclone- and zolpidem-induced increase in the duration of activations saturates at 5 μM, and that both drugs enhance receptor activity via similar mechanisms.

Consistent with our macropatch data, these results suggest rapid drug binding and provide no evidence for low-affinity binding interactions between drug and the receptor.

Activation mechanisms. We recently derived a simple mechanism for activation by GABA using single-channel currents from four synaptic $GABA_A$ receptors, including the α1β2γ2L receptor (Dixon *et al.*, 2014; Figure 7A). This mechanism had previously been validated at α1β2γ2S receptors (Lema and Auerbach, 2006). We re-examined this mechanism on α1β2γ2S $GABA_A$ receptors activated by GABA and modulated by the two benzodiazepine-site ligands. We first used it to fit open and shut dwell histograms across a broad range of GABA concentrations, as previously described (Keramidas and Harrison, 2010; Dixon *et al.*, 2014). This enabled us to obtain state transition rate constants for the GABA-binding and activation steps for this mechanism by maximum likelihood fitting (Colquhoun and Sigworth, 1995; Qin *et al.*, 1997; Table 1). The K_D for GABA (n = 7 data sets, Table 2), was similar to values previously derived for α1-containing $GABA_A$ receptors (Lema and Auerbach, 2006; Dixon *et al.*, 2014). This scheme was then extended to include a single-binding step for eszopiclone and zolpidem (Figure 7B). This binding step was connected to either R, A_1R, A_2R^1 or A_2R^2 separately, and fitted each time to data sets comprised of a series of open and shut dwell histograms (n = 3–4 for each modulator) that included data obtained at 1, 5 and 20 μM drug concentrations (Figure 7C, D). During this fitting, the binding steps for GABA were fixed to those previously determined (K_D = 119 μM), but all other rate constants varied freely. A sequential decrease in the K_D for drug binding was observed when moving from left to right in the mechanism (K_D, $R > A_1R > A_2R^1 > A_2R^2$, Figure 7B). Thus, the two highest affinities were for A_2R^1 and A_2R^2. However, A_2R^2 was rejected as a candidate for drug binding because it produced current simulations that deactivated too slowly relative to the recorded currents (see below), whereas R and A_1R were rejected because corresponding simulated currents were exceedingly fast. When the modulator binding step was connected to A_2R^1 the K_Ds for eszopiclone and zolpidem were calculated to be 83 ± 7 nM and 76 ± 9 nM respectively (Table 1). Other equilibrium constants that notably changed downstream of the binding reaction was Φ (φ'_1/φ'_{-1}), which increased from 1.2 for GABA alone to 2.2 and 2.4 for eszopiclone and zolpidem, respectively; in both cases, the change was significantly different from GABA alone. A comparable inference was made for the same receptors in the presence of diazepam, where an increase in the equilibrium constant between two postulated shut states that preceded channel opening was sufficient to recapitulate a diazepam-induced shift in the concentration–response relationship (Gielen *et al.*, 2012). Our analysis revealed that both modulators also consistently increased Σ (σ_1/σ_{-1}) and decreased E3 (α_3/β_3) (Table 2). However, the effects on Σ and E3 were relatively minor and failed to reach significance.

Ensemble current simulations. Our derived mechanisms for activation by GABA alone, and modulation by eszopiclone and zolpidem were used to simulate ensemble macropatch currents. The activation and deactivation time constants were then compared with those of the corresponding recorded currents, and thereby acted as an independent validation of

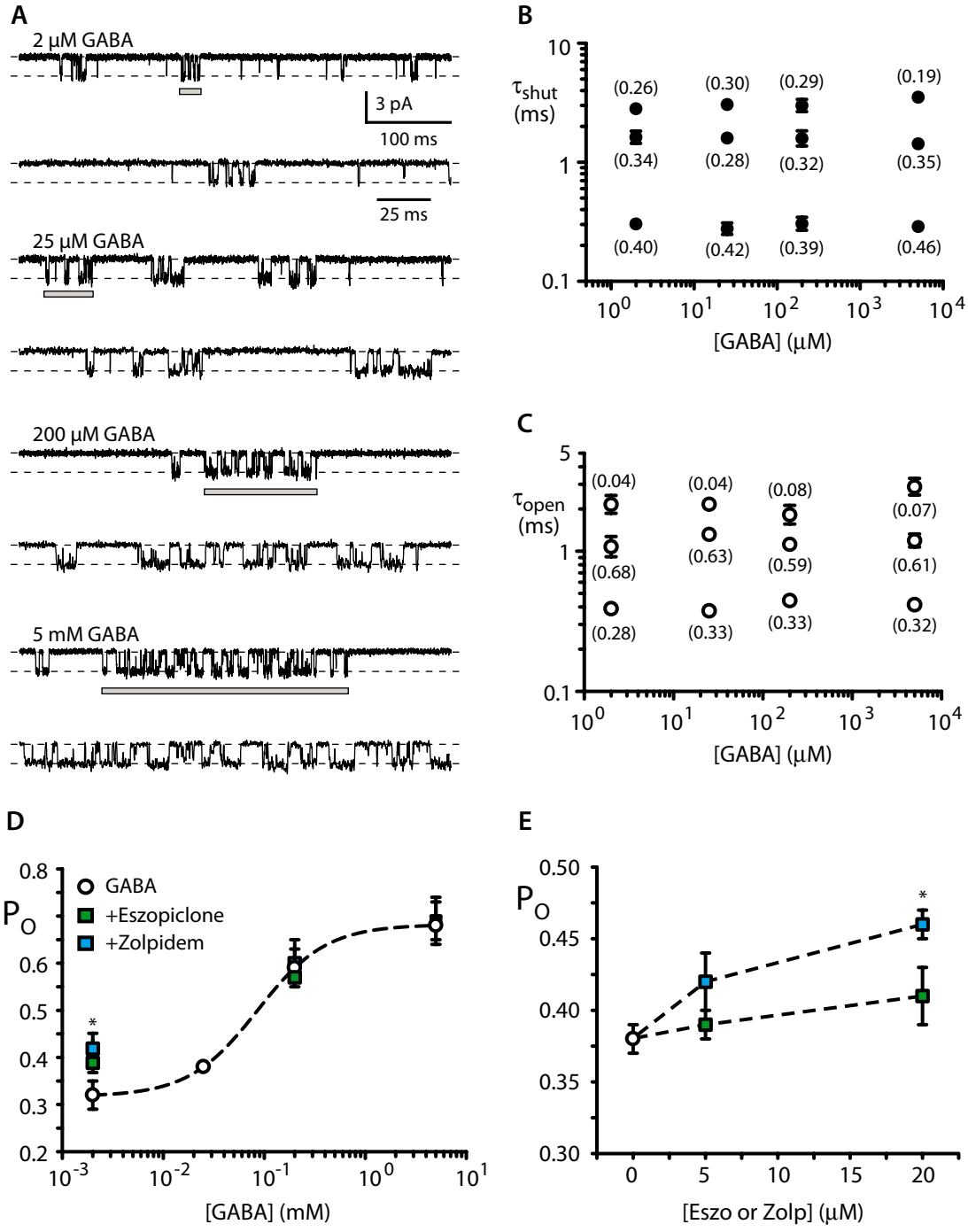

Figure 4

Eszopiclone and zolpidem have modest effects on single-channel open probability. (A) A series of single-channel recordings, showing that single-channel activations increase in duration and intra–activation open probability with increasing GABA concentration. Examples of activations are indicated by a grey horizontal bar and shown in expanded view directly below each trace for each concentration. (B and C) Scatter plots of shut and open dwell components as a function of concentration of GABA. The corresponding fractions are shown in parentheses next to each data point. (D) Group concentration-response plot showing an increase in intra–activation open probability as a function of GABA concentration. 1 μM eszopiclone or zolpidem had no detectable effect on P_o at high GABA concentrations. A modest increase in P_o was seen at 2 μM GABA. The data for GABA alone were fit to a Hill-type equation. (E) The effect on P_o with increasing concentrations of modulator. The concentration of GABA was kept constant at 25 μM for these experiments. Only zolpidem (Zolp) at 20μM increased P_o. *$P < 0.05$, significantly different from GABA alone.

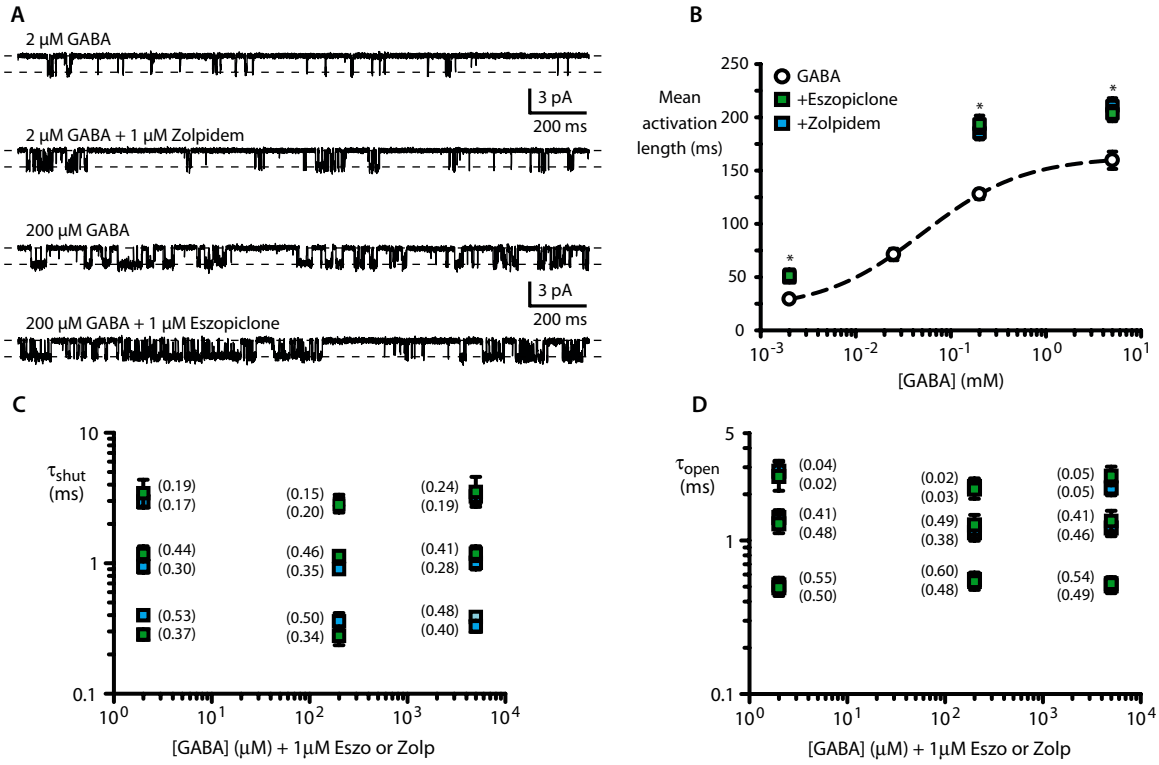

Figure 5

Eszopiclone and zolpidem increase the active periods of single-channel currents. (A) Single-channel recording at 2 μM and 200 μM GABA and 1 μM zolpidem (above) and eszopiclone (below). The upper and lower pairs of traces were recorded from the same patch to control for channel number. Note that both drugs increased the durations of active periods. (B) Group concentration-response plot showing the effects on active period durations in the presence of 1 μM eszopiclone ($n = 7$) or zolpidem ($n = 8$) as a function of GABA concentration. The plot for GABA alone ($n = 11$–14) was fit to a Hill-type equation. *$P < 0.05$, significantly different from GABA alone. (C and D) Scatter plots of shut and open dwell components in the presence of 1 μM zolpidem or eszopiclone. The corresponding fractions for each time constant are shown in parentheses.

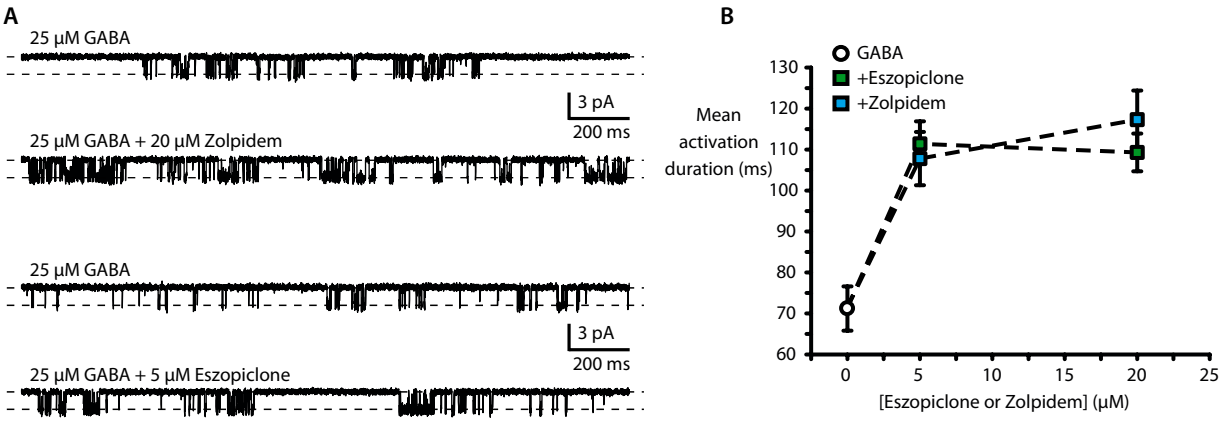

Figure 6

The effects of eszopiclone and zolpidem on activation duration saturate at low μM concentrations. (A) Single-channel currents elicited by 25 μM GABA followed by co-application of 25 μM GABA plus 20 μM zolpidem (above) or 5 μM eszopiclone (below). Each pair of recordings was obtained from the same patch. (B) Group plot showing that eszopiclone ($n = 6$–8) or zolpidem ($n = 4$–6) increased the duration of active periods (GABA; $n = 15$) with similar potency, which saturated at 5 μM drug concentration.

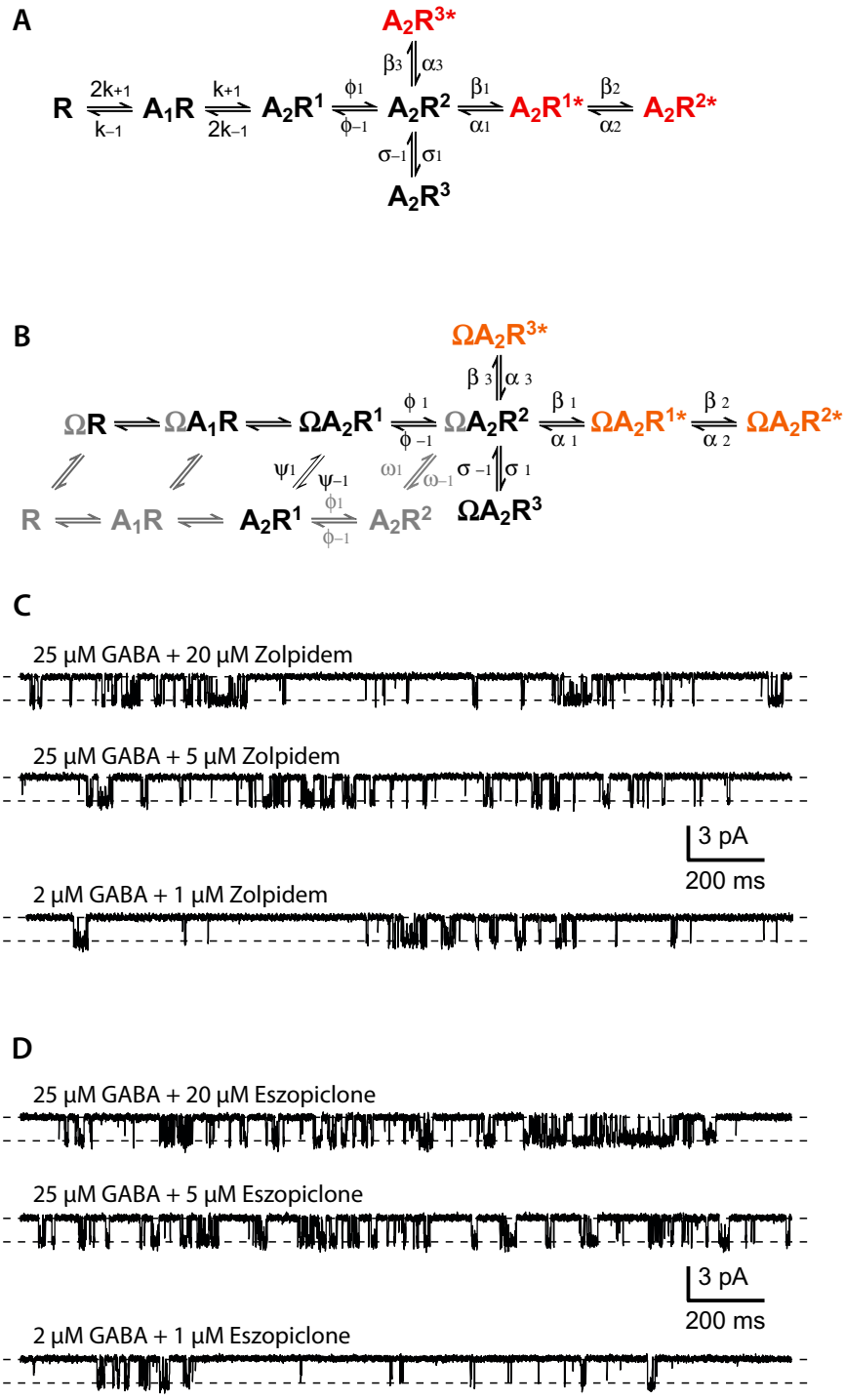

Figure 7

Mechanisms for activation and modulation of α1β2γ2S $GABA_AR$s. (A) A mechanism for activation by GABA, where, A is the agonist and R is the receptor. A_1R and A_2R denote a singly and doubly liganded receptor respectively. The superscripted numbers denote the state number and the asterisk denotes open, conducting states (red). The symbols above and below the double arrows that connect states are the rate constants for the forward and backward transitions. (B) The same activation mechanism as in A with a single-binding step for modulator (eszopiclone or zolpidem) leading to GABA bound and modulator bound (Ω) states. Modulator-bound open states are shown in orange. When the binding step for modulator was appended to the grey states the mechanism did not successfully simulate ensemble currents. An accurate fit to single-channel and ensemble currents was achieved when the modulator was appended to A_2R^1. The scheme used for the simulations is shown in black. (C) Examples of data sets that were used to determine the rate and zolpidem-binding constants in the mechanism shown in B. (D) Examples of data sets that were used to determine the rate and eszopiclone-binding constants in the mechanism shown in B. Note that scheme was fitted (maximum likelihood) to open and shut dwell components of the data shown in C and D to optimize the corresponding rate constants.

Table 1
Rate constants for activation scheme

GABA ($n = 7$)												
k_{+1}	k_{-1}	ϕ_1	ϕ_{-1}	σ_1	σ_{-1}	β_1	α_1	β_2	α_2	β_3	α_3
3.2 ± 0.8[#]	380 ± 38	1110 ± 55	933 ± 73	143 ± 55	348 ± 106	955 ± 70	1911 ± 165	256 ± 66	747 ± 103	961 ± 185	912 ± 146
GABA + eszopiclone ($n = 3–4$)											
ψ_1	ψ_{-1}	ϕ_1'	ϕ_{-1}'	σ_1'	σ_{-1}'	β_1'	α_1'	β_2'	α_2'	β_3'	α_3'
9.7 ± 0.6[$]	8.0 ± 4.0	1802 ± 54	812 ± 32	223 ± 42	351 ± 4	920 ± 59	1972 ± 137	260 ± 43	793 ± 29	950 ± 80	1011 ± 38
GABA + zolpidem ($n = 3–4$)											
ψ_1	ψ_{-1}	ϕ_1'	ϕ_{-1}'	σ_1'	σ_{-1}'	β_1'	α_1'	β_2'	α_2'	β_3'	α_3'
9.9 ± 0.5[#]	7.5 ± 6.0	1816 ± 330	752 ± 209	206 ± 38	362 ± 13	923 ± 180	2008 ± 180	243 ± 177	755 ± 54	934 ± 59	1101 ± 56

Rate constants for state transitions related to the schemes in Figure 7.
[#]$\times 10^6$.
[$]$\times 10^7$.

the mechanisms (Kienker, 1989; Dixon *et al.*, 2014). The activation mechanism for GABA alone produced a simulated current that activated with a time constant of 0.5 ms and deactivated with two exponential components with a weighted average of 9.0 ms. These compare well with the recorded values (Figure 8A), suggesting our mechanism, together with rate constants, provides a good description of α1β2γ2S $GABA_A$ receptor activation by GABA. A similar procedure was carried out for current modulation by eszopiclone and zolpidem. The mechanism used for these simulations included the high-affinity binding step for the modulator, leading to GABA- and modulator-bound states. The eszopiclone-modulated simulation produced a current that activated with a 10–100% rise time constant of 0.4 ms and deactivated with a weighted time constant of 14 ms (Figure 8B). Similarly, the zolpidem-modulated current simulation produced parameters for activation and weighted deactivation that were, 0.4 ms and 15 ms respectively (Figure 8C). The quantitative likeness between simulations and recorded currents suggests that the simple mechanism used here is a suitable descriptor of activation and modulation of $GABA_A$ receptors by benzodiazepine site modulators.

Discussion and conclusions

As with traditional benzodiazepines, zolpidem and eszopiclone work by enhancing GABAergic inhibitory drive. Here we have identified two mechanisms by which they achieve this. Our macropatch results indicate that they potentiate ensemble currents by slowing the deactivation phase. This occurs rapidly, as indicated by the similarity between brief (≤1 ms) and longer (1–2 min) drug applications onto macropatches. Indeed, exposing the receptors to either drug for several minutes in the heterosynapse and primary culture experiments resulted in a similar increase in current decay times. In addition, our results from sIPSCs and mIPSCs show that they not only slow the decay of GABAergic inhibitory postsynaptic currents via a direct action on the postsynaptic $GABA_A$ receptors, they also increase the magnitude of GABAergic synaptic currents, an observation also made for zolpidem on cortical neurons (Perrais and Ropert, 1999). This later effect could be because GABA release at some synapses is sub-saturating. As zolpidem and eszopiclone appear to achieve these effects via identical mechanisms and similar potencies at the α1β2γ2 $GABA_A$ receptors, their differing therapeutic profiles must be due to either different pharmacological effects at other CNS receptors (Jia *et al.*, 2009; Nutt and Stahl, 2010), or to different pharmacokinetic or distribution profiles.

On the level of a single $GABA_A$ receptor, potentiation was manifested mainly as an increase in the duration of activations. An increase in the open-state occupancy was also detected at low GABA concentrations, as also reported for diazepam (Li *et al.*, 2013). The duration of an active period is a key functional property of a channel, and it is this property that correlates with decay times in ensemble currents (Wyllie *et al.*, 1998; Dixon *et al.*, 2014). Although the lowest GABA concentration used here to elicit activations was probably too high (2 μM, activation duration ~30 ms), estimates of the minimum activation duration (~7–10 ms; Macdonald *et al.*,

Table 2

Equilibrium constants

GABA (n = 7)					
K_D-G (μM)	**Φ**	**Σ**	**E1**	**E2**	**E3**
119 ± 24	1.2	0.4	0.5	0.3	1.1
GABA + eszopiclone (n = 3–4)					
K_D-E (nM)	**Φ′**	**Σ′**	**E1′**	**E2′**	**E3′**
83 ± 7	2.2	0.6	0.5	0.3	0.9
GABA + zolpidem (n = 3–4)					
K_D-Z (nM)	**Φ′**	**Σ′**	**E1′**	**E2′**	**E3′**
76 ± 9	2.4	0.6	0.5	0.3	0.8

Equilibrium constants calculated from Table 1. K_D-(G)ABA (k_{-1}/k_{+1}), K_D-E or-Z (ψ_{-1}/ψ_{+1}), Φ (ϕ_1/ϕ_{-1}), Σ (σ_1/σ_{-1}), E1 (α_1/β_1), E2 (β_2/α_2), E3 (β_3/α_3).

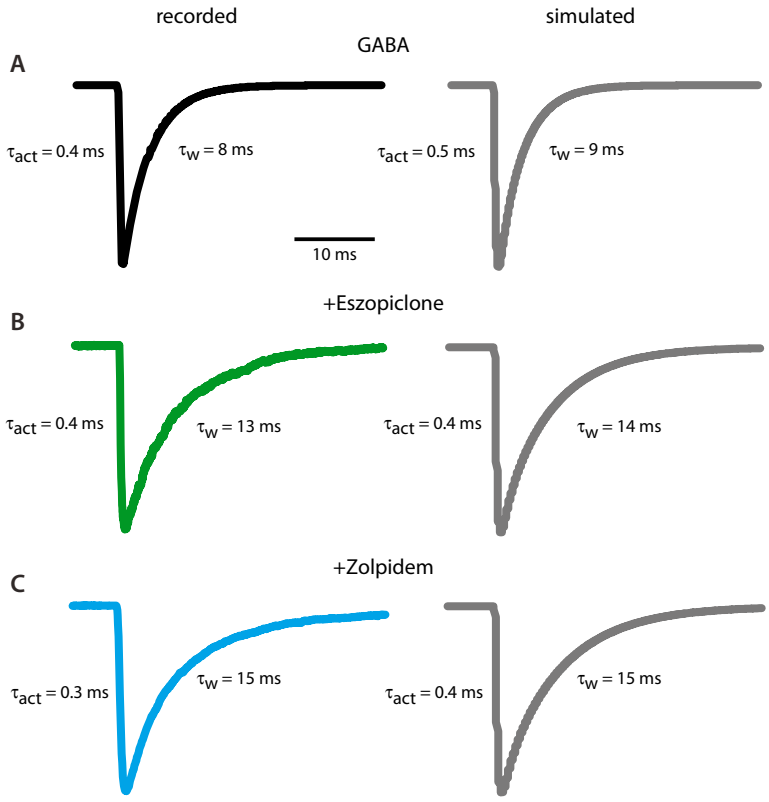

Figure 8

Recorded and simulated ensemble currents. (A) Ultra-fast application of saturating GABA (5 mM) produces a rapidly developing current (0.4 ms) that decays with a weighted time constant of 8 ms (left). Using the mechanism shown in Figure 6A along with the rate constant in Table 1 generates a simulated macropatch current with similar activation and decay times (right). (B) A macropatch current recorded in the presence of eszopiclone (left). The mechanism shown in Figure 6B, along with corresponding rate constants in Table 1 generates a simulated ensemble current (right) that is similar to the recorded current. (C) A macropatch current recorded in the presence of zolpidem (left). As for eszopiclone, the mechanism shown in Figure 6B, together with corresponding rate constants, generates a simulated ensemble current (right) that is similar to the recorded current.

1989; Keramidas *et al.*, 2006) is a much closer match to the decay times of the ensemble currents measured here. We utilized a simple activation mechanism that incorporates six fully liganded states to determine how the benzodiazepine-binding site ligands might induce longer single-channel activations. GABA and drug concentrations that $GABA_A$ receptors at native synapses would be exposed to were used in the experiments. Our scheme omits un- and mono-(GABA) liganded open and shut states, due to the similarity in the shut dwell time analysis across GABA concentrations (Lema and Auerbach, 2006; Keramidas and Harrison, 2010), which indicated that the vast majority of activations arose from fully liganded receptors. However, we acknowledge that below 2 μM GABA there may be some contribution from mono-liganded receptors to the overall activity (Macdonald *et al.*, 1989), and that un-liganded openings, although possible (Colquhoun and Lape, 2012), would be exceedingly rare at the GABA concentrations used here. The mechanism facilitated an estimation of the K_D for each modulator. Our analysis suggests that both modulators bind the receptor with similar affinities (K_Ds ~ 80 nM), similar to estimates obtained from radio-ligand-binding experiments (Hanson *et al.*, 2008). In contrast to mutagenesis studies that suggest that there are some structural differences between zolpidem and eszopiclone, that mediate potentiation (Morlock and Czajkowski, 2011), our study suggests that on a functional level, these two modulators are equivalent. This could occur if the net work (energy changes) performed by zolpidem and eszopiclone on the receptor is similar and independent of the structural details of the ligand and binding site residues (Purohit *et al.*, 2014). This inference is supported by the similar K_Ds for both modulators, which is a function of the energy of binding.

In a previous study using the same activation mechanism as employed here, we reported that channels with a higher affinity for GABA remain active for longer (Dixon *et al.*, 2014). Here, we further report that by promoting the transition between two connected, fully liganded shut states (Φ) that precede channel opening, the activations of a single channel can also be increased. An increase in the equilibrium constant between two pre-conducting states has also been inferred by fitting diazepam concentration–response data with a simple, linear mechanism on the same $GABA_A$ receptors (Gielen *et al.*, 2012). This is in contrast to the conclusions of all other studies that have investigated the mechanisms of benzodiazepine-induced current enhancement. benzodiazepines have generally been modelled as increasing the receptor affinity for GABA (Rogers *et al.*, 1994) or the receptor 'gating' efficacy (the final shut-to-open transition; Rusch and Forman, 2005; Campo-Soria *et al.*, 2006; Li *et al.*, 2013). A crucial difference between other studies and ours is that our single channel and ensemble currents were best described by an activation mechanism that contains two connected shut states that immediately follow ligand binding, and that a discrete binding step for drug was appended to the scheme. Our results are qualitatively similar to those of Gielen *et al.*, albeit with a smaller increase in the pre-conducting transition equilibrium constant. However, our data are also described by a more complex data-derived activation mechanism, based on single-channel analysis. Recording from single $GABA_A$ receptors also enabled us to directly demonstrate the corresponding functional outcome of increasing Φ. The slightly higher Φ value for zolpidem is consistent with a marginally faster activation rate in macropatch currents. Although we found no significant changes in Σ and E3, both drugs affected these constants in the same manner. Other studies identify the shut state associated with Σ as a fast desensitized receptor state (Jones and Westbrook, 1995). The observed increase in the duration of activations in the presence of both drugs is also consistent with a reduced occupancy of the receptor in desensitized states.

Structurally, Φ could represent a concerted movement of all subunits, as has been demonstrated for glycine and ACh receptors (Burzomato *et al.*, 2004; Lape *et al.*, 2008) or two, independent binding site-specific structural changes that are each linked to channel opening in the ACh receptor (Mukhtasimova *et al.*, 2009). These function-based investigations are consistent with local binding site structural changes that do not affect the initial encounter affinity (K_D) for the agonist, but rather underlie the structural switch to a higher affinity state associated with an activated receptor (Purohit and Auerbach, 2013; Purohit *et al.*, 2014). In addition, these studies suggest that for agonists that bind orthosteric sites, such as ACh and GABA, direct communication between binding sites is not required for receptor activation. For modulators such as zolpidem and eszopiclone that bind to a site that is homologous to the orthosteric site, we find that, here too, the current enhancing effects can be wholly accounted for with a constant K_D for GABA. This essentially rules out direct communication between the GABA and zolpidem/eszopiclone-binding sites as a candidate for Φ. High-resolution structures of pLGICs suggest that agonist binding triggers the inner β-sheet of the extracellular domain to rotate while simultaneously tilting the outer β-sheet upwards to close a critical binding site element (loop C) around the bound agonist (Bocquet *et al.*, 2009; Hilf and Dutzler, 2009). These movements are then transmitted to the pore-lining, second (M2) of four transmembrane domains via a differential movement of loops 2 and 7 (both part of the inner β-sheet) and the β10-M1 domain, which is linked to the outer β-sheet.

Alternately, Φ may represent a conformational change restricted to the interface of the ligand binding and transmembrane domains (LBD-TMD). For example, in the α1 homomeric glycine receptor, the magnitude of a conformational change reported by a label attached to a residue on loop 2 (α1A52C) was correlated with agonist efficacy (Pless and Lynch, 2009). This suggests that Φ corresponds to an agonist-specific conformational change at the LBD-TMD interface. This is supported by an earlier study showing that the α1A52S mutation specifically alters Φ in the same glycine receptor (Plested *et al.*, 2007). Moreover, combined crystallography and functional analyses suggest Φ may correspond to a 'locally closed' conformation whereby all receptor domains reside in the activated state with exception of M2 (Prevost *et al.*, 2012; 2013). As this state involves loop 2 and the M2–M3 linker adopting a specific conformation relative to each other, it supports the idea of Φ involving an agonist-specific conformational change at the LBD-TMD interface (Plested *et al.*, 2007; Pless and Lynch, 2009).

As a crucial step in the development of new therapeutics, it is important to have a quantitative understanding of how therapeutic drugs bind to $GABA_A$ receptors and modulate

their activity. A universal mechanism for modelling $GABA_A$ receptor activation and modulation by endogenous and synthetic compounds would facilitate this. Our mechanism may be suitable as a starting point. This mechanism has now been tested on several synaptic $GABA_A$ receptors (Lema and Auerbach, 2006; Dixon *et al.*, 2014), where it successfully accounts for single-channel and ensemble currents, including those modulated by eszopiclone and zolpidem.

Acknowledgements

This study was supported by the Australian Research Council, grant ID – DP120104373. J. W. L. is supported by a fellowship from the National Health and Medical Research Council of Australia.

Author contributions

C. D., N. L. H., J. W. L. and A. K. conceived and designed experiments. C. D. and A. K. performed experiments, analysed the data and drafted the manuscript. C. D., N. L. H., J. W. L. and A. K. edited the manuscript.

Conflict of interest

The authors declare that they have not any conflict of interest.

References

Alexander SPH, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Catterall WA *et al.* (2013). The Concise Guide to PHARMACOLOGY 2013/14: Ligand-Gated Ion Channels. Br J Pharmacol 170: 1582–1606.

Berezhnoy D, Nyfeler Y, Gonthier A, Schwob H, Goeldner M, Sigel E (2004). On the benzodiazepine binding pocket in GABAA receptors. J Biol Chem 279: 3160–3168.

Bocquet N, Nury H, Baaden M, Le Poupon C, Changeux JP, Delarue M *et al.* (2009). X-ray structure of a pentameric ligand-gated ion channel in an apparently open conformation. Nature 457: 111–114.

Burzomato V, Beato M, Groot-Kormelink PJ, Colquhoun D, Sivilotti LG (2004). Single-channel behavior of heteromeric alpha1beta glycine receptors: an attempt to detect a conformational change before the channel opens. J Neurosci 24: 10924–10940.

Campo-Soria C, Chang Y, Weiss DS (2006). Mechanism of action of benzodiazepines on GABAA receptors. Br J Pharmacol 148: 984–990.

Colquhoun D, Lape R (2012). Perspectives on: conformational coupling in ion channels: allosteric coupling in ligand-gated ion channels. J Gen Physiol 140: 599–612.

Colquhoun D, Sigworth FJ (1995). Fitting and statistical analysis of single-channel records. In: Sakmann B, Neher E (eds). Single-Channel Recordings, 2nd edn. Plenum Press: New York, NY.

Darcourt G, Pringuey D, Salliere D, Lavoisy J (1999). The safety and tolerability of zolpidem – an update. J Psychopharmacol 13: 81–93.

Dixon C, Sah P, Lynch JW, Keramidas A (2014). GABAA receptor alpha and gamma subunits shape synaptic currents via different mechanisms. J Biol Chem 289: 5399–5411.

Gielen MC, Lumb MJ, Smart TG (2012). Benzodiazepines modulate GABAA receptors by regulating the preactivation step after GABA binding. J Neurosci 32: 5707–5715.

Hanson SM, Morlock EV, Satyshur KA, Czajkowski C (2008). Structural requirements for eszopiclone and zolpidem binding to the gamma-aminobutyric acid type-A (GABAA) receptor are different. J Med Chem 51: 7243–7252.

Hilf RJ, Dutzler R (2009). Structure of a potentially open state of a proton-activated pentameric ligand-gated ion channel. Nature 457: 115–118.

Jia F, Goldstein PA, Harrison NL (2009). The modulation of synaptic GABA(A) receptors in the thalamus by eszopiclone and zolpidem. J Pharmacol Exp Ther 328: 1000–1006.

Jones MV, Westbrook GL (1995). Desensitized states prolong GABAA channel responses to brief agonist pulses. Neuron 15: 181–191.

Keramidas A, Harrison NL (2010). The activation mechanism of alpha1beta2gamma2S and alpha3beta3gamma2S GABAA receptors. J Gen Physiol 135: 59–75.

Keramidas A, Moorhouse AJ, Schofield PR, Barry PH (2004). Ligand-gated ion channels: mechanisms underlying ion selectivity. Prog Biophys Mol Biol 86: 161–204.

Keramidas A, Kash TL, Harrison NL (2006). The pre-M1 segment of the alpha1 subunit is a transduction element in the activation of the GABAA receptor. J Physiol 575 (Pt 1): 11–22.

Kienker P (1989). Equivalence of aggregated Markov models of ion-channel gating. Proc R Soc Lond B Biol Sci 236: 269–309.

Korpi ER, Grunder G, Luddens H (2002). Drug interactions at GABA(A) receptors. Prog Neurobiol 67: 113–159.

Krystal AD, Walsh JK, Laska E, Caron J, Amato DA, Wessel TC *et al.* (2003). Sustained efficacy of eszopiclone over 6 months of nightly treatment: results of a randomized, double-blind, placebo-controlled study in adults with chronic insomnia. Sleep 26: 793–799.

Lape R, Colquhoun D, Sivilotti LG (2008). On the nature of partial agonism in the nicotinic receptor superfamily. Nature 454: 722–727.

Lema GM, Auerbach A (2006). Modes and models of GABA(A) receptor gating. J Physiol 572 (Pt 1): 183–200.

Li P, Eaton MM, Steinbach JH, Akk G (2013). The benzodiazepine diazepam potentiates responses of alpha1beta2gamma2L gamma-aminobutyric acid type A receptors activated by either gamma-aminobutyric acid or allosteric agonists. Anesthesiology 118: 1417–1425.

Macdonald RL, Rogers CJ, Twyman RE (1989). Kinetic properties of the GABAA receptor main conductance state of mouse spinal cord neurones in culture. J Physiol 410: 479–499.

Morlock EV, Czajkowski C (2011). Different residues in the GABAA receptor benzodiazepine binding pocket mediate benzodiazepine efficacy and binding. Mol Pharmacol 80: 14–22.

Mukhtasimova N, Lee WY, Wang HL, Sine SM (2009). Detection and trapping of intermediate states priming nicotinic receptor channel opening. Nature 459: 451–454.

Nutt DJ, Stahl SM (2010). Searching for perfect sleep: the continuing evolution of GABAA receptor modulators as hypnotics. J Psychopharmacol 24: 1601–1612.

Pawson AJ, Sharman JL, Benson HE, Faccenda E, Alexander SP, Buneman OP *et al.*; NC-IUPHAR (2014). The IUPHAR/BPS Guide to PHARMACOLOGY: an expert-driven knowledge base of drug targets and their ligands. Nucl. Acids Res 42 (Database Issue): D1098–1106.

Perrais D, Ropert N (1999). Effect of zolpidem on miniature IPSCs and occupancy of postsynaptic GABAA receptors in central synapses. J Neurosci 19: 578–588.

Pless SA, Lynch JW (2009). Magnitude of a conformational change in the glycine receptor beta1–beta2 loop is correlated with agonist efficacy. J Biol Chem 284: 27370–27376.

Plested AJ, Groot-Kormelink PJ, Colquhoun D, Sivilotti LG (2007). Single-channel study of the spasmodic mutation {alpha}1A52S in recombinant rat glycine receptors. J Physiol 581 (Pt 1): 51–73.

Prevost MS, Sauguet L, Nury H, Van Renterghem C, Huon C, Poitevin F *et al.* (2012). A locally closed conformation of a bacterial pentameric proton-gated ion channel. Nat Struct Mol Biol 19: 642–649.

Prevost MS, Moraga-Cid G, Van Renterghem C, Edelstein SJ, Changeux JP, Corringer PJ (2013). Intermediate closed state for glycine receptor function revealed by cysteine cross-linking. Proc Natl Acad Sci U S A 110: 17113–17118.

Purohit P, Auerbach A (2013). Loop C and the mechanism of acetylcholine receptor-channel gating. J Gen Physiol 141: 467–478.

Purohit P, Bruhova I, Gupta S, Auerbach A (2014). Catch-and-hold activation of muscle acetylcholine receptors having transmitter binding site mutations. Biophys J 107: 88–99.

Qin F, Auerbach A, Sachs F (1997). Maximum likelihood estimation of aggregated Markov processes. Proc Biol Sci 264: 375–383.

Rogers CJ, Twyman RE, Macdonald RL (1994). Benzodiazepine and beta-carboline regulation of single GABAA receptor channels of mouse spinal neurones in culture. J Physiol 475: 69–82.

Rudolph U, Knoflach F (2011). Beyond classical benzodiazepines: novel therapeutic potential of GABAA receptor subtypes. Nat Rev Drug Discov 10: 685–697.

Rusch D, Forman SA (2005). Classic benzodiazepines modulate the open-close equilibrium in alpha1beta2gamma2L gamma-aminobutyric acid type A receptors. Anesthesiology 102: 783–792.

Wingrove PB, Safo P, Wheat L, Thompson SA, Wafford KA, Whiting PJ (2002). Mechanism of alpha-subunit selectivity of benzodiazepine pharmacology at gamma-aminobutyric acid type A receptors. Eur J Pharmacol 437: 31–39.

Wyllie DJ, Behe P, Colquhoun D (1998). Single-channel activations and concentration jumps: comparison of recombinant NR1a/NR2A and NR1a/NR2D NMDA receptors. J Physiol 510 (Pt 1): 1–18.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13142

Appendix S1 Additional methods, definitions and open and shut dwell histograms for GABA alone, GABA with eszopiclone and GABA with zolpidem.

Figure S1 Open and shut dwell histograms. (A) Dwell histograms for 2 μM GABA. (B) Dwell histograms for 2 μM GABA + 1 μM eszopiclone. (C) Dwell histograms for 2 μM GABA + 1 μM zolpidem.