Zolpidem is a potent stoichiometry-selective modulator of $\alpha_1\beta_3$ GABA$_A$ receptors: evidence of a novel benzodiazepine site in the $\alpha_1$-$\alpha_1$ interface

Ahmad Tarmizi Che Has$^1$, Nathan Absalom$^1$, Petra S. van Nieuwenhuijzen$^1$, Andrew N. Clarkson$^{1,2}$, Philip K. Ahring$^1$ & Mary Chebib$^1$

Zolpidem is not a typical GABA$_A$ receptor hypnotic. Unlike benzodiazepines, zolpidem modulates tonic GABA currents in the rat dorsal motor nucleus of the vagus, exhibits residual effects in mice lacking the benzodiazepine binding site, and improves speech, cognitive and motor function in human patients with severe brain injury. The receptor by which zolpidem mediates these effects is not known. In this study we evaluated binary $\alpha_1\beta_3$ GABA$_A$ receptors in either the $3\alpha_1:2\beta_3$ or $2\alpha_1:3\beta_3$ subunit stoichiometry, which differ by the existence of either an $\alpha_1$-$\alpha_1$ interface, or a $\beta_3$-$\beta_3$ interface, respectively. Both receptor stoichiometries are readily expressed in Xenopus oocytes, distinguished from each other by using GABA, zolpidem, diazepam and Zn$^{2+}$. At the $3\alpha_1:2\beta_3$ receptor, clinically relevant concentrations of zolpidem enhanced GABA in a flumazenil-sensitive manner. The efficacy of diazepam was significantly lower compared to zolpidem. No modulation by either zolpidem or diazepam was detected at the $2\alpha_1:3\beta_3$ receptor, indicating that the binding site for zolpidem is at the $\alpha_1$-$\alpha_1$ interface, a site mimicking the classical $\alpha_1$-$\gamma_2$ benzodiazepine site. Activating $\alpha_1\beta_3$ ($3\alpha_1:2\beta_3$) receptors may, in part, mediate the physiological effects of zolpidem observed under distinct physiological and clinical conditions, constituting a potentially attractive drug target.

$\gamma$-aminobutyric acid receptors of type A (GABA$_A$) are members of the Cys-loop family of ligand-gated ion channels that mediate most of the inhibitory neurotransmission in the central nervous system (CNS). These receptors are pentameric assemblies of individual subunits including $\alpha_1$-$6$, $\beta_1$-$3$, $\gamma_1$-$3$, $\delta$, $\epsilon$, $\pi$ and $\theta$. The majority of receptors are composed of $\alpha$, $\beta$, and $\gamma$ or $\delta$ subunits$^{1-4}$. Depending on the subunit composition, the receptors are located either at the synapse ($\alpha_1/2/3\beta\gamma$ receptors) where they are exposed to brief (ms) bursts of high concentrations of GABA when the neuron fires (termed phasic inhibition) or at extrasynaptic regions ($\alpha_1/4/6\beta\gamma$, $\alpha_1\beta\gamma$, or $\alpha_1\beta\delta$ receptors) where receptors typically experience long lasting (min to hours) exposure to relatively low but consistent concentrations of GABA (termed tonic inhibition)$^{1-4}$. The most abundant receptors are $\alpha_1\beta\gamma$ receptors, and these are activated and modulated by a variety of pharmacologically and clinically unrelated agents including benzodiazepines, barbiturates, anaesthetics and neurosteroids, all of which bind at distinct binding sites located within the receptor complex$^{1-4}$.

Zolpidem is an imidazopyridine, a non-benzodiazepine, that binds with high-affinity to $\alpha_1$ containing GABA$_A$ receptors ($\alpha_1\beta\gamma$) and with lower affinity to $\alpha_2$ or $\alpha_3$ containing receptors ($\alpha_2\beta\gamma$ and $\alpha_3\beta\gamma$)$. In contrast, zolpidem is significantly more efficacious at $\alpha_2$ or $\alpha_3$ than $\alpha_1$ containing receptors$. The binding site for zolpidem at these receptors is located at the interface between the principle side (+) of the $\alpha$ and the complementary side (−) of $\gamma_2$ subunit$^{10,11}$. When zolpidem binds to this site, there is a structural change to the protein

---

$^1$Faculty of Pharmacy, The University of Sydney, Sydney, New South Wales, 2006, Australia. $^2$Department of Anatomy, Brain Health Research Centre and Brain Research New Zealand, School of Medical Sciences, University of Otago, Dunedin, 9054, New Zealand. Correspondence and requests for materials should be addressed to M.C. (email: mary.collins@sydney.edu.au)
complex resulting in an increase in GABA potency. The effects of zolpidem can be competitively blocked by the neutral modulator flumazenil.

Like many benzodiazepines, zolpidem exhibits sedative and hypnotic effects via α1 containing receptors, but unlike benzodiazepines, zolpidem reverses cognitive and motor deficits in neurophysiological states, improves motor function in patients with Parkinson’s disease, progressive supranuclear palsy and stroke. Studies also show that there are residual effects with zolpidem in the C57BL/6 mouse where binding by zolpidem to α1 + β2(−) is attenuated. Further, zolpidem can modulate tonic GABA currents in the rat dorsal motor nucleus of the vagus and primary motor cortex, implicating extrasynaptic receptors containing α5 and δ subunits. However, these receptors do not significantly respond to zolpidem and neither do γ1/3 containing receptors.

Thus we sought to determine whether binary α3 receptors are potential targets that mediate zolpidem’s atypical effects.

Binary α3 receptors lack the benzodiazepine α-γ2 interface and co-exist with classical GABA, Re23–25. The physiological role of these receptors remains elusive, due to a lack of pharmacological tools that differentiate these receptors from their ternary counterparts. Interestingly, there are reports that certain benzodiazepines are able to enhance GABA actions at these receptors by binding to sites other than the classical α-γ2 interface.

In pentameric α3 receptors, the third subunit is replaced with either an α1 or a β3 subunit leading to two distinct receptors that differ in subunit stoichiometry, 2α:3β3 or 3α:2β3. The consequence of this is that 3α:2β3 receptors contain an α-α interface whereas 2α:3β receptors contain a β-β interface, that are clearly distinct. Drugs that selectively bind to either the α-α or β-β interface will have stoichiometric selective effects as demonstrated by the in vivo and in vitro effects of drugs at the related nicotinic acetylcholine α4/32 receptors.

In this study, we evaluated the effects of GABA, Zn2+, zolpidem and diazepam at binary α1/3 GABA receptors. Receptors were expressed as 3α:2β3 and 2α:3β3 subunit stoichiometries in Xenopus oocytes using two electrode voltage clamp electrophysiology. Both receptors expressed readily, and were distinguished from each other by their pharmacology. The 2α:3β3 receptor was highly sensitive to GABA and Zn2+, while the 3α:2β3 receptor was less sensitive to GABA and Zn2+. Interestingly, zolpidem and diazepam were potent allosteric modulators of 3α:2β3 receptors and the effects were attenuated by flumazenil. While zolpidem displayed high efficacy at the 3α:2β3 stoichiometry, the efficacy of diazepam was significantly lower. No modulation by either zolpidem or diazepam was detected at 2α:3β3 receptors. These results demonstrate that the α1-1 face of the β3 interface contains a binding site for zolpidem mimicking the classical α1-2 benzodiazepine site. This novel target may explain why zolpidem enhances tonic GABA currents as binary α3 receptors are reported to be extrasynaptic, and may be a target for its motor effects after severe brain damage.

Results
Different α1/3 receptors are expressed by injecting Xenopus oocytes with different cRNA ratios.

The assembly of Cys-loop receptors in Xenopus laevis oocytes into specific stoichiometries can be directed by injecting cRNA with variant subunit ratios. To determine whether varying the injection ratios led to the expression of different receptors, we injected five mixtures of α1 and β3 cRNAs into oocytes (α1:β3 in 1:1, 1:10, 1:30 and 1:100 ratios). The total amount of cRNA injected ranged from 2.5 to 4.0 ng/oocyte and the functional properties of receptors including holding currents, GABA sensitivity and maximum peak current amplitudes were compared.

Initially, receptors expressed by the most extreme injection ratios 1:1 and 30:1, were compared. The holding currents of oocytes injected with α1 + β3 (1:1) cRNA were −150 ± 93 nA, n = 6 when voltage-clamped at −60 mV, suggesting constitutive receptor activity (Fig. 1A). In contrast, oocytes injected with α1 + β3 (30:1) had negligible holding current levels that averaged −13 ± 10 nA, n = 9 (Fig. 1A; p < 0.05).

GABA activated receptors expressed from both injection ratios in a concentration-dependent manner, with maximum similar peak-current amplitudes ranging from 1200 to 4200 nA (p > 0.05). Fitting peak-current amplitudes as a function of the GABA concentration to the Hill equation revealed higher GABA sensitivity with receptors from the 1:1 cRNA ratio compared to those formed from the 30:1 ratio. The derived EC50 values were significantly different (p < 0.0001) with a 10-fold difference between receptors at the 1:1 and 30:1 ratios (2.8 μM and 26 μM, respectively) (Fig. 1B; Table 1). The GABA sensitivity from the 30:1 injection ratio was similar to that of the ubiquitous α1/3β3-2 receptor (Fig. 1B; Table 1).

In contrast, receptors formed from 5:1, 10:1, and 20:1 cRNA injection ratios resulted in less uniform receptor populations. This resulted in intermediate GABA sensitivities, concentration-response curves with shallow Hill slopes and comparatively high standard errors for individual data points (data not shown). Thus, receptors formed from different injection ratios had different functional properties that are likely the result of the expression of distinct receptor populations with altered subunit stoichiometries.

Expressing stoichiometry specific α1/3 receptors using concatenated constructs.

To determine whether data obtained by varying cRNA ratios are from uniform receptor populations that consist of 2α:3β and 3α:2β stoichiometries, the functional properties of receptors expressed with a concatenated construct linking the β3 and α1 subunits were compared to receptors expressed with the 1:1 or 30:1 cRNA injection ratios. For this experiment, the N-terminal of the α1 subunit was linked to the C-terminal of the β3 subunit to generate the β3-α1 concatenated construct as previously described. When cRNA transcribed from the β3-α1 construct was injected into oocytes (2.5 ng/oocyte) no GABA-elicted currents were observed (n = 10). Therefore, to express receptors in a 2α:3β and 3α:2β stoichiometry, β3-α1 was co-injected with either the β3 or α1 subunit, respectively.

First, the properties of 2α:3β receptors were compared to receptors formed by the 1:1 injection ratio. Both holding current levels and the GABA sensitivities of oocytes expressing receptors from the concatenated β3-α1 + β3 (1:2) construct were similar to oocytes injected with free α1 + β3 (1:1) subunits, with holding currents
of $-153 \pm 65$ nA, $n = 6$ compared to $-150 \pm 93$ nA, $n = 6$ when voltage-clamped at $-60$ mV. The constitutive current observed with both $\alpha_1 + \beta_3 (1:1)$ and $\beta_3 - \alpha_1 + \beta_3 (1:2)$ could be the result of homomeric $\beta_3$ receptors being expressed. To determine whether or not the observed holding currents are due to homomeric $\beta_3$ receptors, we evaluated oocytes expressing $\alpha_1 + \beta_3 (1:1)$ and $\beta_3 - \alpha_1 + \beta_3 (1:2)$ using histamine (1 mM), an agonist that activates only homomeric $\beta_3$ receptors. We found that all cells that exhibited a significant holding current injected with either $\alpha_1 + \beta_3 (1:1)$ (4 out of 4 cells) or $\beta_3 - \alpha_1 + \beta_3 (1:2)$ (1 out of 4 cells) had some response to histamine (data not shown), indicating that homomeric $\beta_3$ receptors are the major contributor to the holding current. Irrespectively, GABA activated expressed receptors in a concentration-dependent manner, with maximum peak-current amplitudes ranging from 1000 to 2000 nA. The GABA EC$_{50}$ values of 41 $\mu$M and 26 $\mu$M from $\beta_3 - \alpha_1 + \alpha_1 (1:2)$ and $\alpha_1 + \beta_3 (30:1)$ injections were similar ($p > 0.05$, Fig. 1B,C, Table 1).
**Table 1.** GABA concentration response relationships at various GABA<sub>A</sub> receptors. Xenopus laevis oocytes were injected with cRNA mixtures containing the indicated GABA<sub>A</sub> receptor subunits. Background subtracted peak current amplitudes for full GABA concentration-response curves in presence or absence of zolpidem were fitted to the Hill equation (fixed bottom of 0 and slope of 1) using non-linear regression and normalized to the maximal fitted value. Averaged normalized data points were next fitted to the Hill equation and resultant EC<sub>50</sub> values and maximal efficacies (E<sub>max</sub>) are presented as mean with 95% confidence intervals for n experiments.

| Receptor      | cRNA ratio | EC<sub>50</sub> (μM) | E<sub>max</sub> (%) | n  |
|---------------|------------|-----------------------|---------------------|----|
| α1+β3+γ2      | 1:1:1      | 1.5 (1.2–1.8)         | 94 (91–101)         | 7  |
| α1+β3         | 1:3        | 2.6 (2.1–3.2)         | 96 (92–100)         | 9  |
| α1+β3+γ2      | 3:1        | 3.3 (4.5–6.1)         | 99 (96–102)         | 5  |
| α1+β3+γ2      | 3:1:5      | 5.6 (4.5–7.0)         | 97 (93–100)         | 5  |
| α1+β3+γ2      | 1:1:3      | 1.5 (12–15)           | 98 (96–100)         | 5  |
| α1+β3         | 1:3:1      | 1.2 (1.2–1.8)         | 98 (94–101)         | 6  |
| α1+β3+γ2      | 1:1:5      | 3.3 (30:1)            | 91 (87–95)          | 7  |
| α1+β3+γ2      | 1:1:3      | 7.1 (5.7–9.0)         | 91 (87–95)          | 7  |
| α1+β3+γ2      | 1:2:3      | 3.3 (30:1)            | 94 (91–101)         | 6  |
| α1+β3+γ2      | 1:1:5      | 7.1 (5.7–9.0)         | 91 (87–95)          | 7  |

Thus, there were no observable differences in the functional properties of α1β3 receptors in either the 2α1:3β2 or 3α1:2β3 stoichiometries between receptors expressed by different subunit ratios or a concatenated construct (Fig. 1D). Although the chosen cRNA ratios for the individual subunits appeared sufficient to ensure predominately uniform receptor populations, the use of concatenated receptors may potentially yield more uniform populations. However, this methodology inherently carries a risk that the physical linkage of subunits could affect receptor pharmacology. Hence, for the remainder of the manuscript, conclusions will be drawn from data generated using both methodologies.

**α1β3 receptors show stoichiometry-specific sensitivity to Zn<sup>2+</sup> ions.** Zn<sup>2+</sup> ions are used to differentiate α1β3 from α1β3γ2 GABA<sub>A</sub> receptors. Both α1-β3 and α3-β3 interfaces contribute to the binding pockets for Zn<sup>2+</sup> 40. Since 3α1:2β3 receptors do not contain a β3-α3 interface, sensitivity to Zn<sup>2+</sup> will likely be altered as a result of differing subunit stoichiometries. Therefore, Zn<sup>2+</sup> inhibition of α1β3 receptor currents elicited by GABA (at EC<sub>50</sub> concentrations) was measured in oocytes.

Zn<sup>2+</sup> (10 μM) inhibited 87 ± 10% (n = 5) of the GABA-induced current at 2α1:3β3 receptors formed by injection of α1 + β3 (1:1) cRNA (Fig. 2A). In contrast, only 13 ± 10% (n = 6) inhibition was observed at receptors formed by injecting α1 + β3 (30:1). Next, inhibitory concentration-response relationships were performed to ascertain the potency of Zn<sup>2+</sup> at each stoichiometry. At the 2α1:3β3 stoichiometry, Zn<sup>2+</sup> inhibited receptors from the 1:1 ratio with an IC<sub>50</sub> value of 0.84 μM (Fig. 2B). A similar IC<sub>50</sub> value of 1.6 μM for Zn<sup>2+</sup> inhibition at concatenated receptors was observed with α1β3 + β3 (1:2) injection (Fig. 2C). These two IC<sub>50</sub> values were not significantly different from each other (p > 0.05). At the highest concentration of Zn<sup>2+</sup>, both receptors were fully inhibited.

At the 3α1:2β3 receptors, the maximal tested concentration of Zn<sup>2+</sup> (100 μM) only displayed partial inhibition (26 ± 17%, n = 6) at receptors from the 30:1 ratio (Fig. 2B). Similar partial inhibition was observed using the concatenated β3-α1 + α1 (1:2) construct (Fig. 2C). In both cases, the level of inhibition was too low to allow for meaningful fitting to the Hill equation. The partial inhibition of Zn<sup>2+</sup> at 3α1:2β3 receptors mimicked observations at α1β3γ2 receptors (Fig. 2B,C).

These data demonstrate that receptors with a β3-α3 interface have high sensitivity to inhibition by Zn<sup>2+</sup> (Fig. 2D). While it was previously suggested that residues located in the α1-β3 interface also contribute to Zn<sup>2+</sup> sensitivity 40 this appears to require substantially higher concentrations. In addition, α1β3γ2 receptors that also lack the β3-α3 interface, were likewise relatively insensitive to Zn<sup>2+</sup> inhibition at concentrations below 100 μM.

**Zolpidem is a positive modulator of α1β3 with a 3α1:2β3 subunit stoichiometry.** The α1-α1 interface of 3α1:2β3 receptors is homologous to the α1-γ2 interface that binds benzodiazepines and non-benzodiazepines. Zolpidem has in vivo effects that are not related to binding in the classical α1-γ2 benzodiazepine site, and we wanted to evaluate whether this α1 preferring modulator displayed any efficacy at α1β3 possessing an α1-α1 interface. Zolpidem (1 μM) was co-applied with a low GABA concentration (~EC<sub>50</sub>) to evaluate potential modulation of control currents at 3α1:2β3 and compared with 2α1:3β3 and α1β3γ2 receptors.

As expected, zolpidem (1 μM) had no effect at 2α1:3β3 receptors expressed by a 1:1 ratio of free subunits (Fig. 3A). In contrast, 3α1:2β3 receptors expressed from the 30:1 ratio were positively modulated by zolpidem. This resulted in an increase in current amplitudes of more than 100% compared with the current elicited by the GABA alone. This enhancement was inhibited by co-application of ilumazenil (1 μM), a benzodiazepine site neutral antagonist (Fig. 3B). As expected, zolpidem (1 μM) also enhanced GABA-elicted currents at α1β3γ2 receptors, an enhancement that could be inhibited by ilumazenil (Fig. 3C).

To determine the potency of zolpidem potentiation at 3α1:2β3 receptors, full concentration-response relationships were obtained and compared with those from the α1β3γ2 receptor. When injecting free subunits using α1 + β3 (30:1) and α1 + β3 + γ2 (1:1:5) cRNA, zolpidem had a significantly lower EC<sub>50</sub> value of 0.10 μM at α1β3 receptors compared to an EC<sub>50</sub> value of 0.48 μM at α1β3γ2 (p < 0.01) (Fig. 3D; Table 2). However, when injecting cRNA containing the concatenated construct using β3-α1 + α1 (1:2) and β3-α1 + γ2 (1:2), the EC<sub>50</sub> value...
of 0.030 μM at α1β3 receptors was not significantly different to the EC50 value of 0.050 μM at α1β3γ2 receptors (p > 0.05) (Fig. 3E; Table 2). Efficacy levels at α1β3 receptors were 120% and 110% for the 30:1 ratio and concatenated receptors, respectively. Higher values were observed at α1β3γ2 receptors with 340% and 350% at α1+β3+γ2 (1:1:5) vs. β3-α1+γ2 (1:2), respectively. Hence zolpidem modulated GABA-evoked currents at 3α1β3 receptors. Importantly, the potency of zolpidem at α1β3 (3α1:1β3) receptors was comparable with α1β3γ2 receptors regardless of whether free subunits or concatenated subunits were injected. While the potency of zolpidem was similar at both receptors, these experiments suggest that zolpidem enhanced α1β3γ2 receptors with greater efficacy. However, in this type of assay efficacy of a modulator is highly dependent on the utilized GABA concentration and should be treated with caution.

Modulatory mechanism of action of zolpidem at α1β3 receptors possessing a 3α1:2β3 subunit stoichiometry. The hallmark feature of allosteric modulation via the α1-γ2 benzodiazepine site by e.g. diazepam and zolpidem is an increase in the apparent potency of GABA. This causes a shift in the GABA concentration-response curve to the left in presence of zolpidem with little accompanying change in the maximal current amplitudes. In order to assess how zolpidem affects 3α1β3 stoichiometry, GABA concentration-response relationships were measured in the presence of zolpidem and compared to that in its absence.
At 3α1+β3 receptors expressed by injecting α1+β3 (30:1) cRNA, zolpidem (1 μM) left-shifted the GABA concentration-response curve by decreasing the EC50 value from 26 μM to 5.6 μM (Fig. 4A, Table 1). Hence, zolpidem modulation caused a significant 5-fold change (p < 0.0001) of the GABA potency with no observed change in the maximum GABA-evoked peak current amplitudes. At receptors obtained by the concatenated β3-α1 (1:2) construct, zolpidem (1 μM) likewise caused a significant (p < 0.0001) 6-fold change in the potency of GABA, with an increase in the EC50 value from 41 μM to 7.1 μM (Fig. 4B, Table 1). At α1+β3γ2 receptors, zolpidem (10 μM)
significantly decreased the EC_{50} value from 53 \mu M to 13 \mu M (Fig. 4C; Table 1; p < 0.0001) with no change in the maximum peak current amplitudes. These data demonstrate that the mechanism of modulatory action by zolpidem is increase of the GABA potency at both \( \alpha_1 \beta_3 \) and \( \alpha_1 \beta_3 \gamma_2 \) receptors. This increase ranged from 4 to 6 fold, which is in agreement with previous observations at \( \alpha_1 \beta_3 \gamma_2 \) receptors.\(^8\) The modulatory effect was not accompanied by any change in maximal GABA-evoked peak current amplitudes and the similar magnitude of the changes in EC_{50} values suggest that zolpidem has a similar efficacy at the two receptor types. Although zolpidem is not structurally a classical benzodiazepine, it binds at a similar site within the \( \alpha_1 \gamma_2 \) of \( \alpha_1 \beta_3 \gamma_2 \) receptors as benzodiazepines. Taken together, zolpidem is most likely binding at the \( \alpha_1 \beta_3 \gamma_2 \) interface of \( \alpha_1 \beta_3 \gamma_2 \) receptors to modulate receptor function analogous to the modulation of receptor function via binding at the \( \alpha_1 \gamma_2 \) interface (Fig. 4D).

Diazepam enhances GABA currents at \( \alpha_1 \beta_3 \) receptors with a 3\( \alpha_1:2\beta_3 \) subunit stoichiometry albeit with low efficacy. Finally, we determined whether \( \alpha_1 \beta_3 \) receptors could be modulated by the classical benzodiazepine diazepam, which binds with equal affinity to GABA\( _3 \) receptors containing \( \alpha_1, \alpha_2, \alpha_3 \) and \( \alpha_5 \) subunits.\(^{41,42}\) Like zolpidem, diazepam (1 \mu M) enhanced GABA-induced currents at \( \alpha_1 \beta_3 \) receptors obtained with \( \alpha_1 + \beta_3 \) (30:1) cRNA and this effect was inhibited by co-application of flumazenil (Fig. 5A). However, the enhancement by diazepam was low in comparison with zolpidem (Fig. 5B). A full concentration-response relationship revealed an EC_{50} value for diazepam of 0.040 \mu M (Fig. 5C). A similar EC_{50} value of 0.020 \mu M was estimated for diazepam modulation of concatenated receptors and the observed potencies...
Subunit stoichiometry were differentiated from each other using GABA, Zn\(^{2+}\) (1:1) (data not shown).

Analogous to zolpidem, diazepam (1 \(\mu M\)) resulted in 51% modulation and these were not significantly different from each other (Fig. 5C; \(p > 0.05\)). This represents half or less of the modulation observed with zolpidem (Table 2). Use of free subunit cRNA diazepam enhanced GABA-elicited currents by a maximum of 40% whereas use of concatenated subunits resulted in 51% modulation and these were not significantly different from each other (Fig. 5C; \(p > 0.05\)). This represents half or less of the modulation observed with zolpidem (Table 2). An analogous to zolpidem, diazepam (1 \(\mu M\)) did not change the maximal GABA-evoked current amplitudes at 2\(\alpha1:3\beta3\) receptors obtained by injecting \(\alpha1 + \beta3\) (1:1) (data not shown).

Discussion

Binary GABA\(_A\) receptors co-exist with their ternary counterparts and are gaining attention for their sensitivity to certain benzodiazepines. When evaluating the pharmacology of binary receptors the concept of stoichiometry-specific binding sites becomes important, because each receptor stoichiometry will have a specific binding interface that will contribute to a distinct pharmacological profile for that receptor. For pentameric \(\alpha1\beta3\) receptors, the subunit position, normally occupied by a \(\gamma2\) subunit in \(\alpha1\beta3\gamma2\) receptors, is substituted with either an \(\alpha1\) or a \(\beta3\) subunit leading to two distinct receptors, that differ by the presence of either an \(\alpha1-\alpha1\) or a \(\beta3-\beta3\) interface (Fig. 1D). Pharmacologically targeting either the \(\alpha1-\alpha1\) or \(\beta3-\beta3\) interface leads to stoichiometric selective effects as demonstrated by the \textit{in vivo} and \textit{in vitro} effects of drugs at the related nicotinic acetylcholine \(\alpha4\beta2\) receptors. In this study, we demonstrate that binary \(\alpha1\beta3\) receptors composed of \(2\alpha1:1\beta3\) or \(3\alpha1:2\beta3\) subunit stoichiometry were differentiated from each other using GABA, Zn\(^{2+}\), zolpidem and diazepam. Two methodologies were used to express the two receptor stoichiometries (2\(\alpha1:3\beta3\) or 3\(\alpha1:2\beta3\)) in \textit{Xenopus laevis} oocytes. In the first method, we injected cRNA mixtures with variant subunits ratios, causing relative overexpression of one subunit versus the other, and hence, increasing the likelihood of receptor assembly with more of the overexpressed subunit. While this is an efficient method, it cannot always ensure uniform receptor populations or rule out the formation of “obscure” receptor populations such as 4\(\alpha1\beta1\) receptors. In the second method, concatenated \(3\beta3-\alpha1\) subunits were used, ensuring that linkers force the assembly process. While this is efficient for creating uniform receptor populations, the presence of linkers may affect receptor pharmacology. Nevertheless, we found that both methods enabled the assembly of \(\alpha1\beta3\) receptors, which were indistinguishable in their pharmacology using either methodology and concluded the receptors to be either 2\(\alpha1:3\beta3\) or 3\(\alpha1:2\beta3\) receptors. This contrasted the original study using concatenated \(3\beta3-\alpha1\) subunits and individual \(\alpha1\) or \(\beta2\) subunit mRNA. It is not clear why Baumann and colleagues did not see functional receptors when using \(3\beta3-\alpha1\) and individual \(\alpha1\) subunits. However the study used a 1:1 injection ratio while we used a 2-fold excess of \(\alpha1\) subunit mRNA. Further we used \(3\beta3\) subunit and not \(\beta2\) and there may be differences in the formation of receptors depending on the type of \(\beta\) subunit.
Xenopus oocytes expressing α1β3 (2α1:3/33) receptors consistently displayed voltage-clamp holding-current levels constituting approximately 10% of maximal GABA-evoked peak current amplitudes. This suggests the existence of homomeric β3 receptors contributing to the holding current as histamine was able to activate cells that exhibited constitutive currents.28,29 In contrast, expression of 3α1:2β3 receptors did not result in levels significantly different from un-injected control oocytes. However, the potency of GABA differed approximately 10-fold between 2α1:1/33 and 3α1:2/33 receptors with 2α1:1/33 displaying the highest GABA sensitivity. The lower GABA sensitivity at 3α1:2/33 receptors was not significantly different to the “classical” α1β3γ2 receptor indicating “normal” receptor function.

Previous studies demonstrated that Zn²⁺ inhibits binary GABA_{α1} β3 receptor function by an allosteric mechanism and this action is critically dependent on the composition of α1 and β3 subunits.40,44 This is consistent with our data showing that binary GABA_{α1} receptors can be differentiated by Zn²⁺, showing that 2α1:3β3 receptors are easily blocked by Zn²⁺ while 3α1:2β3 receptors are not. Thus, the presence of an extra β3 subunit, and thereby a β3-β3 interface, confers the resulting binary receptors with several unique characteristics such as, higher sensitivity for GABA and higher sensitivity for Zn²⁺ inhibition. The high sensitivity to Zn²⁺ inhibition is intriguing given their extrasynaptic location. Physiological brain concentrations of Zn²⁺ can reach up to 100 μM, i.e. the sweet-spot for completely inhibiting 2α1:3β3 receptors, and one could speculate that these receptors may in part be responsible for a Zn²⁺-regulated tonic current.

In contrast, binary receptors with an α1-α1 interface have more resemblance to ternary α1β3γ2 receptors. They exhibit a lower degree of constitutive activity, and lower sensitivity for GABA and Zn²⁺ inhibition. Furthermore, we demonstrate that zolpidem efficiently modulates GABA activity at 3α1:2β3 but not 2α1:1/3β3 receptors with a binding site for zolpidem most likely at the α1-α1 interface. Several lines of evidence point to zolpidem’s actions occurring via the α1-α1 interface of the 3α1:2β3 receptor rather than in the potential of zolpidem via binding to the classical α1-γ2 benzodiazepine site: i) Like zolpidem, diazepam is a positive allosteric modulator of 3α1:2β3 receptors albeit with lower efficacy levels; ii) the effects of zolpidem and diazepam are inhibited by the antagonist flumazenil at a concentration normally used to inhibit benzodiazepines at the α1-γ2 benzodiazepine site; and iii) the modulatory effect of zolpidem at 3α1:2β3 receptors was one of increasing the potency of GABA without significant change to maximal current amplitudes. These data comprise signature features of modulatory actions by benzodiazepines via the classical α1-γ2 interface benzodiazepine site40,41, however, as such a site is not available in 2α1:1/3β3 receptors, zolpidem’s actions must occur via the α1-α1 interface. Interestingly, the potency of zolpidem at 3α1:2β3 receptors was equal to or marginally higher than that observed at α1β3γ2 receptors suggesting that the observed effects could have clinical relevance.

In addition to phasic currents, zolpidem modulates tonic GABA currents in the rat dorsal motor nucleus of the vagus nerve and primary motor cortex.42 This is intriguing as the subunit composition of zolpidem-sensitive extrasynaptic receptors is unlikely to be due to α5, γ1/3 or δ containing GABA_{α1} receptors, as such receptors do not significantly respond to zolpidem.43 Recently, α1β3γ2 receptors were reported to exist at extrasynaptic sites44 but the residual effects of zolpidem in γ2F771 knockin mice excludes these receptors as a target for zolpidem. Although non-GABA_{α1} receptors may also contribute to zolpidem’s effects, binary α1β3γ2 receptors represent 10% of the total number of extrasynaptic receptors in hippocampal pyramidal neurons.45 Thus binary α1β3 receptors that possess an α1-α1 subunit interface may contribute to the residual zolpidem effects reported in γ2F771 knockin mice that lack zolpidem-sensitive α1-α1 interfaces.19,36,51

Binary α1β3 γ2 receptors that are positively modulated by zolpidem may also have significant clinical effects in a number of neurological conditions where the ratio and location of various GABA receptor subunits are altered, for example, stroke52–54, or epilepsy55,56. Under these conditions, dramatic changes are occurring in the brain. After stroke the α1 mRNA significantly increases55–54, resulting in an increase in α1 protein.52 At the same time β-subunit mRNA decreases, and thus may affect the formation of GABA_{α1} receptors in a way that the subunit composition of the receptors is altered favouring receptors that possess an α1-α1 subunit interface. In order to ascertain that the increase in α1 protein after stroke results in a change in receptor function that mimics the pharmacology of binary α1β3 γ2 receptors possessing an α1-α1 interface, further work including patch clamp recordings are required.

In corroboration, there are increasing clinical reports documenting that zolpidem is not a typical hypnotic, improving cognitive and motor function in human patients with severe brain injury. For instance, acute administration of zolpidem in a patient suffering from brain injury resulted in a transient improvement in aphasia57. Brefel-Courbon and colleagues (2007) reported improved motor performance and neuropsychological status in a patient who was given zolpidem58. In addition, zolpidem has benefits on motor disorders in patients with Parkinson’s disease13–15, has a transient improvement of spinocerebellar ataxia16, and improve symptoms of progressive supranuclear palsy17. Whilst flumazenil inhibits the effect of zolpidem indicating a GABA_{α1} receptor is involved with these patients, classical benzodiazepines are not very effective in improving speech or motor function59 indicating an unusual GABA_{α1} receptor to be the target.

Similar positive modulation is seen by zolpidem at α1-α1 and α1-γ2 interfaces, however the complete molecular interactions involving ligand binding and the accompanying conformational changes to the receptor complex still needs to be resolved. While the principal face (+) of the α1 subunit is the same in the two sites, the complementary face (−) of α1 and γ2 are obviously different. Although the actions of zolpidem, diazepam and flumazenil suggest substantial overlap between the two sites, other compound structures could potentially be fully selective for the α1-α1 interface. Future studies are required to ascertain the structure-function requirements for this interface.

In conclusion, this study demonstrates GABA_{α1β3} receptors can express in either 2α1:1/33 and 3α1:2β3 stoichiometry possessing either a β3-β3 or α1-α1 interface, respectively. This difference leads to stoichiometry-specific binding sites, fundamentally changing receptor pharmacology. Indeed, zolpidem at clinically relevant concentrations enhanced GABA actions at 3α1:2β3 but not 2α1:1/3β3 receptors by binding to the
α1-α1 interface in a manner mimicking its actions at the classical benzodiazepine site. These receptors may be the molecular target for zolpidem's described extrasynaptic and clinical effects and constitute a potentially interesting drug target for neurological conditions (e.g. stroke and epilepsy) where the α1 mRNA and protein levels are significantly upregulated.

**Methods**

**Chemicals.** GABA (4-aminobutanoic acid), flumazenil (Ethyl 8-fluoro-5-methyl-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate), pyruvate, theophylline, gentamycin and zinc chloride were obtained from Sigma Aldrich, Sydney, Australia. Diazepam was obtained from Apin Chemicals Ltd, Oxon, UK, and zolpidem (N,N-dimethyl-2-(6-methyl-2-p-tolylimidazo[1,2-a]-pyridin-3-yl) acetamide) from Chemieliva Pharmaceutical, Chongqing, China. Tricaine was purchased from Western Chemical, USA.

**GABA<sub>4</sub> receptor subunit cRNA.** Human α1 and γ2 cDNA subcloned in pCDM8 and 33 in pGEMHE were linearized with the appropriate restriction endonucleases (NotI for α1 and γ2, Nhel for 33 subunit, respectively). Concatenated 33-α1 subunits were developed as previously described<sup>3,4</sup>, subcloned in pN35α and linearized with NotI. cRNA was produced from linearized plasmids using the ‘mMessage mMachine’ T7 transcript kit from Ambion (Austin, TX, USA) as previously described<sup>4,6</sup>. A total of 2–4 ng of cRNA was injected per oocyte. When using free subunits of α1, 33 and γ2, cRNAs were mixed in variant ratios. To ensure incorporation of a free subunit in the pentameric complex, concatenated 33-α1 subunit cRNA was injected with either α1 or γ2 cRNAs in a ratio of 1:2. However, to avoid any potential formation of 33 homomeric receptors concatenated 33-α1 subunit cRNA was injected with 33 in a 1:1 ratio.

**Xenopus oocyte extraction and preparation.** All procedures were using *Xenopus laevis* frogs were approved by the animal ethics committee of The University of Sydney (AEC No. 2013/5269) and are in accordance to the National Health and Medical Research Council (NHMRC) of Australia. In brief, *Xenopus laevis* were anaesthetized using 0.2% w/v solution of tricaine methanesulfonate or tricaine-S (Western Chemical, USA). A transverse incision of about 3 mm in length was made through the outer layer of her skin on the lateral ventral surface. Another incision was made through the connective tissue and muscle layer to reach the ovary wall. A section of the ovary was carefully removed onto the surface of the frog. A small section of ovary was separated and transferred to a tube containing the OR2 solution (82.5 mM NaCl, 5 mM HEPES, 2 mM MgCl₂, and 2 mM KCl; pH 7.4). Oocytes were separated manually from their follicles and digested using 40 mg collagenase A diluted in 15 ml OR2 solution at 18°C for about 1 hour until the oocytes were fully detached from the follicles and the ovary tissue.

Stage V-VI oocytes were injected with 50.6 nl cRNA solution composed of GABA<sub>4</sub>Rs subunit cRNAs in the required ratio. The injected oocytes were incubated in ND96 solution (96 mM NaCl, 5 mM HEPES, 2 mM MgCl₂, 1 mM KCl and 1.8 mM CaCl₂; pH 7.4) supplemented with 2.5 mM Na-pyruvate, 0.5 mM theophylline, 50 mg/ml gentamycin and 50 mg/ml tetracycline for 2–5 days at 18°C.

**Two-electrode voltage-clamp electrophysiology.** The electrophysiological experiments were performed by the two-electrode voltage clamp technique. The membrane potential was measured using an Oocyte Clamp OC-725C amplifier (Warner Instruments Corp, CT, USA) by the voltage electrode with current simultaneously injected by the current electrode to maintain the potential difference across the oocyte membrane at −60 mV. Data were acquired with a LabChart v. 3.5.2 analogue to digital converter and currents low-pass-filtered at 1 kHz and sampled at 3 kHz were measured offline with LabChart v. 3.5.2 software. The bath solution contained the ND96 solution and electrodes were filled with solution of 3 M KCl and 18 mM KCl and 1.8 mM CaCl₂; pH 7.4) at 18°C.

**Data Analysis.** For Zn<sup>2+</sup> inhibition studies, control currents (I<sub>control</sub>) were evoked using a GABA concentration corresponding to −EC<sub>50</sub> and inhibition by Zn<sup>2+</sup> was evaluated by co-applications with GABA<sub>control</sub>. Averaged Zn<sup>2+</sup> inhibition in the presence of GABA EC<sub>50</sub> were depicted as means ± S.E.M. as a function of the Zn<sup>2+</sup> concentration and fitted to the Hill equation by non-linear regression. Enhancement of GABA-gated Cl<sup>−</sup> currents was measured by co-applying modulators with a GABA concentration that elicited 5% of the maximal current, EC<sub>50</sub> are expressed as mean ± S.E.M. of the Hill Coefficient. EC<sub>50</sub> Values are expressed as mean with 95% confidence intervals and Hill coefficients (n<sub>H</sub>) are expressed as mean ± S.E.M.

Concentration–response curves were generated and the data fitted by non-linear regression analysis using GraphPad Prism software (version 5.0). Statistical significance was calculated using unpaired Student t-test with a confidence interval of p < 0.05 to compare parameters derived from individual experiments and data given as mean with 95% confidence intervals from at least 5 oocytes and at least 2 different batches. The logEC<sub>50</sub> derived from individual comparisons, were used for statistical comparisons.

**References**

1. Chebib, M. & Johnston, G. A. GABA-Activated ligand gated ion channels: medicinal chemistry and molecular biology. J Med Chem 43, 1427–1447 (2000).
2. Farrant, M. & Nusser, Z. Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. Nat Rev Neurosci 6, 215–229 (2005).
3. Sieghart, W. Structure, pharmacology, and function of GABA<sub>4</sub> receptor subtypes. Adv Pharmacol 54, 231–263 (2006).
4. Sieghart, W. Allosteric modulation of GABA<sub>4</sub> receptors via multiple drug-binding sites. Adv Pharmacol 72, 53–96 (2015).
5. Sancar, F., Ericksen, S. S., Kucken, A. M., Teissere, J. A. & Czajkowski, C. Structural determinants for high-affinity zolpidem binding to GABA<sub>4</sub> receptors. Mol Pharmacol 71, 38–46 (2007).
6. Hanson, S. M., Morlock, E. V., Satyshur, K. A. & Czajkowski, C. Structural requirements for eszopiclone and zolpidem binding to the gamma-aminobutyric acid type A (GABA\(_A\)) receptor are different. J Med Chem 51, 7243–7252 (2008).
7. Baur, R. & Sigel, E. Replacement of histidine in position 105 in the alpha(5) subunit by cysteine stimulates zolpidem sensitivity of alpha(5)beta(2)gamma(2) GABA\(_A\) receptors. J Neurochem 103, 2556–2564 (2007).
8. Ramerstorfer, J., Furttmuller, R., Vogel, E., Huck, S. & Sieghart, W. The point mutation gamma 2F77I changes the potency and efficacy of benzodiazepine site ligands in different GABA\(_A\) receptor subtypes. Eur J Pharmacol 636, 18–27 (2010).
9. Sanna, E. et al. Comparison of the effects of zalepon, zolpidem, and triazolam at various GABA\(_A\) receptor subtypes. Eur J Pharmacol 451, 103–110 (2002).
10. Sigel, E. Mapping of the benzodiazepine site on GABA\(_A\) receptors. Curr Topics Med Chem 2, 833–839 (2002).
11. Ernst, M., Brauchart, D., Boresch, S. & Sieghart, W. Comparative modeling of GABA\(_A\) receptors: limits, insights, future developments. Neurosci 119, 933–943 (2003).
12. Patat, A. et al. Flumazenil antagonizes the central effects of zolpidem, an imidazopyridine hypnotic. Clin Pharmacol Ther 56, 430–436 (1994).
13. Chen, Y. Y., Sy, H. N. & Wu, S. L. Zolpidem improves akinesia, dystonia and dyskinesia in advanced Parkinson’s disease. J Clin Neurophysiol 15, 955–956 (2008).
14. Hall, S. D. et al. GABA-mediated changes in inter-hemispheric beta frequency activity in early-stage Parkinson’s disease. Neurosci Lett 481, 68–76 (2010).
15. Prokic, E. J. et al. Cortical oscillatory dynamics and benzodiazepine-site modulation of tonic inhibition in fast spiking interneurons. Neuropharmacology 95, 192–205 (2015).
16. Claus, R., Sathshek, M. & Nel, W. Transient improvement of spinocerebellar ataxia with zolpidem. New Eng J Med 351, 511–512 (2004).
17. Chang, A. Y. & Weirich, E. Trial of Zolpidem, Eszopiclone, and Other GABA Agonists in a Patient with Progressive Supranuclear Palsy. Case Rep Med 2014, 107064 (2014).
18. Cope, D. W. et al. Loss of zolpidem efficacy in the hippocampus of mice with the GABA\(_A\) receptor gamma2 F77I point mutation. Eur J Neurosci 21, 3002–3016 (2005).
19. May, A. C., Fleischer, W., Klethe, O., Haas, H. L. & Sergeeva, O. A. Benzodiazepine-site pharmacology on GABA\(_A\) receptors in histaminergic neurons. Br J Pharmacol 170, 222–232 (2013).
20. Gao, H. & Smith, B. N. Zolpidem modulation of phasic and tonic GABA currents in the rat dorsal motor nucleus of the vagus. Neuropeptides 58, 1220–1227 (2010).
21. Khom, S. et al. Pharmacological properties of GABA\(_A\) receptors containing gamma1 subunits. Mol Pharmacol 69, 640–649 (2006).
22. Buht, A. & Sigel, E. A point mutation in the gamma2 subunit of gamma-aminobutyric Acid\(_A\) receptors results in altered benzodiazepine binding site specificity. Proc Nat Acad Sci USA 94, 8824–8829 (1997).
23. Brickley, S. G., Cull-Candy, S. G. & Farrant, M. Single-channel properties of synaptic and extrasynaptic GABA\(_A\) receptors suggest differential targeting of receptor subtypes. J Neurosci 19, 2960–2973 (1999).
24. Sieghart, W. & Sperk, G. Subunit composition, distribution and function of GABA\(_A\) receptor subtypes. Curr Topics Med Chem 2, 795–816 (2002).
25. Mortensen, M. & Smart, T. G. Extrasynaptic alpha5 subunit GABA\(_A\) receptors on rat hippocampal pyramidal neurons. J Physiol 577, 841–856 (2006).
26. Bencsits, E., Ebert, V., Tretter, V. & Sieghart, W. A significant part of native gamma-aminobutyric Acid\(_A\) receptors containing alpha4 subunits do not contain gamma or delta subunits. J Biol Chem 274, 19613–19616 (1999).
27. Sinkkonen, S. T., Luscher, B., Luddens, H. & Korpi, E. R. Autoradiographic imaging of altered synaptic alphabeta2gamma2 and extrasynaptic alphabeta GABA\(_A\) receptors in a genetic mouse model of anxiety. Neurochem Int 44, 539–547 (2004).
28. Walters, R. J., Hadley, S. H., Morris, K. D. & Amin, J. Benzodiazepines act on GABA\(_A\) receptors via two distinct and separable mechanisms. Nat. Neurosci. 3, 1274–1281 (2000).
29. Baur, R. et al. Covalent modification of GABA\(_A\) receptor isoforms by a diazepam analogue provides evidence for a novel benzodiazepine binding site that prevents modulation by these drugs. J Neurochem 106, 2353–2363 (2008).
30. Ramerstorfer, J. et al. The GABA\(_A\) receptor alpha subunit – beta interface: a novel target for subtype selective drugs. J Neurosci 31, 870–877 (2011).
31. Tretter, V., Ehya, N., Fuchs, K. & Sieghart, W. Stoichometry and assembly of a recombinant GABA\(_A\) receptor subunit. J Neurosci 17, 2728–2737 (1997).
32. Farrar, S. J., Whiting, P. J., Bonnett, T. P. & McKernan, R. M. Stoichiometry of a ligand-gated ion channel determined by fluorescence energy transfer. J Biol Chem 274, 10100–10104 (1999).
33. Boileau, A. I., Pearce, R. A. & Czajkowski, C. Tandem subunits effectively constrain GABA\(_A\) receptor stoichiometry and recapitulate receptor kinetics but are insensitive to GABA\(_A\) receptor-associated protein. J Neurosci 25, 11219–11230 (2005).
34. Harpse, K. et al. Unraveling the high- and low-sensitivity agonist responses of nicotinic acetylcholine receptors. J Neurosci 31, 10759–10766 (2011).
35. Ahling, P. K. et al. Engineered alphabeta2 nicotinic acetylcholine receptors as models for measuring agonist binding and effect at the orthosteric low-affinity alpha4-beta4 interface. Neuropharmacol 92, 135–145 (2015).
36. Chua, H. C., Absalom, N. L., Hanrahan, J. R., Viswas, R. & Chebib, M. The Direct Actions of GABA, 2′-Methoxy-6′-Methyllavone and General Anaesthetics at beta3gamma2L GABA\(_A\) Receptors: Evidence for Receptors with Different Subunit Stoichiometries. PloS one 10, e0141359 (2015).
37. Kaur, K. H., Baur, R. & Sigel, E. Unanticipated structural and functional properties of beta-subunit-containing GABA\(_A\) receptors. J Biol Chem 284, 7889–7896 (2009).
38. Sarasa, A. et al. Histamine action on vertebrate GABA\(_A\) receptors: direct channel gating and potentiation of GABA responses. J Biol Chem 283, 10470–10475 (2008).
39. Hörbelt, P. et al. Mutagenesis and computational docking studies support the existence of a histamine binding site at the extracellular beta3 + beta2 interface of homologomeric beta GABA\(_A\) receptors. Neuropharmacol 108, 252–263 (2016).
40. Hosie, A. M., Dunne, E. L., Harvey, R. J. & Smart, T. G. Zinc-mediated inhibition of GABA\(_A\) receptors: discrete binding sites underlie subtype specificity. Nat. Neurosci. 6, 362–369 (2003).
41. Pritchett, D. B. & Seeburg, P. H. Gamma-aminobutyric acidA receptor alpha 5-subunit creates novel type II benzodiazepine receptor pharmacology. J Neurochem 54, 1802–1804 (1990).
42. Wieland, H. A. & Luddens, H. Four amino acid exchanges convert a diazepam-insensitive, inverse agonist-prefering GABA\(_A\) receptor into a diazepam-prefering GABA\(_A\) receptor. J Med Chem 37, 4576–4580 (1994).
43. Baumann, S. W., Baur, R. & Sigel, E. Subunit arrangement of gamma-aminobutyric AcidA type A receptors. J Biol Chem 276, 36275–36280 (2001).
44. Smart, T. G., Moss, S. J., Xie, X. & Huganir, R. L. GABA receptors are differentially sensitive to zinc: dependence on subunit composition. Br J Pharmacol 103, 1837–1839 (1991).
45. Frederikson, C. J. et al. Synaptic release of zinc from brain slices: factors governing release, imaging, and accurate calculation of concentration. J Neurosci Methods 154, 19–29 (2006).
46. Frederikson, C. J. et al. Concentrations of extracellular free zinc (pZn) in the central nervous system during simple anesthetization, ischemia and reperfusion. Experimental neurology 198, 285–293 (2006).
47. Qian, J. & Noebels, J. L. Visualization of transmitter release with zinc fluorescence detection at the mouse hippocampal mossy fibre synapse. *J Physiol* **566**, 747–758 (2005).
48. Vogt, K., Mellor, J., Tong, G. & Nicoll, R. The actions of synaptically released zinc at hippocampal mossy fiber synapses. *Neuron* **26**, 187–196 (2000).
49. Patel, B., Bright, D. P., Mortensen, M., Frolund, B. & Smart, T. G. Context-Dependent Modulation of GABA<sub>A</sub>R-Mediated Tonic Currents. *J Neurosci* **36**, 607–621 (2016).
50. Cope, D. W. et al. Enhanced tonic GABA<sub>A</sub> inhibition in typical absence epilepsy. *Nature medicine* **15**, 1392–1398 (2009).
51. Cope, D. W. et al. Abolition of zolpidem sensitivity in mice with a point mutation in the GABA<sub>A</sub> receptor gamma2 subunit. *Neuropharmacol* **47**, 17–34 (2004).
52. Khodr, E. A., Downey, K. L., Jukkola, P. I., Grayson, D. R. & Kelly, K. M. Expression of GABA<sub>A</sub> receptor alpha1 subunit mRNA and protein in rat neocortex following photofromic infarction. *Brain Res* **1210**, 29–38 (2008).
53. Liu, J. et al. Quantitative reverse transcription-polymerase chain reaction of GABA<sub>A</sub> receptor alpha1, beta1 and gamma2S subunits in epileptic rats following photofromic infarction of neocortex. *Epilepsy Res* **52**, 85–95 (2002).
54. Hui, T. et al. Enhanced phasic GABA inhibition during the repair phase of stroke: a novel therapeutic target. *Brain* **139** (Pt 2), 468–80 (2016).
55. Raol, Y. H. et al. Enhancing GABA<sub>A</sub> alpha 1 subunit levels in hippocampal dentate gyrus inhibits epilepsy development in an animal model of temporal lobe epilepsy. *J Neurosci* **26**, 11342–11346 (2006).
56. Raol, Y. H. et al. Increased GABA<sub>A</sub> receptor alpha1-subunit expression in hippocampal dentate gyrus after early-life status epilepticus. *Epilepsia* **47**, 1665–1673 (2006).
57. Cohen, L., Chaaban, B. & Habert, M. O. Transient improvement of aphasia with zolpidem. *New Eng J Med* **350**, 949–950 (2004).
58. Brefel-Courbon, C. et al. Clinical and imaging evidence of zolpidem effect in hypoxic encephalopathy. *Ann Neurol* **62**, 102–105 (2007).
59. Clauss, R. P. et al. Cerebral blood perfusion after treatment with zolpidem and flumazenil in the baboon. *Arzneimittel-Forschung* **52**, 740–744 (2002).
60. Karim, N. et al. 2′-Methoxy-6-methylflavone: A novel anxiolytic and sedative with subtype selective activating and modulating actions at GABA<sub>A</sub> receptors. *Br J Pharmacol* **165**, 880–896 (2012).
61. Karim, N. et al. 3-Hydroxy-2′-methoxy-6-methylflavone: A potent anxiolytic with a unique selectivity profile at GABA<sub>A</sub> receptor subtypes. *Biochem Pharmacol* **82**, 1971–1983 (2011).

**Acknowledgements**

This work was funded by the National Health and Medical Research Council of Australia (NHMRC) Project Grant 1081733. A.T.C.H was financially supported by the Ministry of Higher Education of Malaysia and Department of Neurosciences, University of Science, Malaysia (USM). P.K.A was co-funded by Imk Almene Fond, Denmark.

**Author Contributions**

A.T.C.H. -Designed and performed experiments; contributed to writing of main text; N.A. -Designed experiments; contributed to writing of main text; P.S.v.N. -contributed to writing of main text; A.N.C. -Designed experiments; contributed to writing of main text; P.K.A. -Designed experiments; contributed to writing of main text; M.C. -Designed experiments; contributed writing of main text; All authors reviewed the manuscript.

**Additional Information**

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Che Has, A. T. et al. Zolpidem is a potent stoichiometry-selective modulator of α1β3 GABA<sub>A</sub> receptors: evidence of a novel benzodiazepine site in the α1-α1 interface. *Sci. Rep.* **6**, 28674; doi: 10.1038/srep28674 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/