



Article

# High-Dose Benzodiazepines Positively Modulate GABA<sub>A</sub> Receptors via a Flumazenil-Insensitive Mechanism

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**Abstract:** Benzodiazepines (BZDs) produce versatile pharmacological actions through positive modulation of GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs). A previous study has demonstrated that high concentrations of diazepam potentiate GABA currents on the  $\alpha_1\beta_2\gamma_2$  and  $\alpha_1\beta_2$  GABA<sub>A</sub>Rs in a flumazenil-insensitive manner. In this study, the high-concentration effects of BZDs and their sensitivity to flumazenil were determined on synaptic ( $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$ ,  $\alpha_5\beta_2\gamma_2$ ) and extra-synaptic ( $\alpha_4\beta_2\delta$ ) GABA<sub>A</sub>Rs using the voltage-clamp electrophysiology technique. The in vivo evaluation of flumazenil-insensitive BZD effects was conducted in mice via the loss of righting reflex (LORR) test. Diazepam induced biphasic potentiation on the  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$  and  $\alpha_5\beta_2\gamma_2$  GABA<sub>A</sub>Rs, but did not affect the  $\alpha_4\beta_2\delta$  receptor. In contrast to the nanomolar component of potentiation, the second potentiation elicited by micromolar diazepam was insensitive to flumazenil. Midazolam, clonazepam, and lorazepam at 200  $\mu$ M exhibited similar flumazenil-insensitive effects on the  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$  and  $\alpha_5\beta_2\gamma_2$  receptors, whereas the potentiation induced by 200  $\mu$ M zolpidem or triazolam was abolished by flumazenil. Both the GABA<sub>A</sub> antagonist pentylentetrazol and Fa173, a proposed transmembrane site antagonist, abolished the potentiation induced by 200  $\mu$ M diazepam. Consistent with the in vitro results, flumazenil antagonized the zolpidem-induced LORR, but not that induced by diazepam or midazolam. Pentylentetrazol and Fa173 antagonized the diazepam-induced LORR. These findings support the existence of non-classical BZD binding sites on certain GABA<sub>A</sub>R subtypes and indicate that the flumazenil-insensitive effects depend on the chemical structures of BZD ligands.

**Keywords:** GABA<sub>A</sub> receptors; benzodiazepine; non-classical binding site; voltage-clamp electrophysiology; loss of righting reflex



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## 1. Introduction

GABA<sub>A</sub> receptors (GABA<sub>A</sub>Rs), the major inhibitory neurotransmitter receptors in the nervous system, are complex receptors that are critically involved in numerous physiological and pathological processes. They are heterogeneous pentamers assembled from at least 19 subunits ( $\alpha_{1-6}$ ,  $\beta_{1-3}$ ,  $\gamma_{1-3}$ ,  $\delta$ ,  $\epsilon$ ,  $\pi$ ,  $\rho_{1-3}$ , and  $\theta$ ), and there are potentially dozens of active GABA<sub>A</sub>R subtypes with distinct distributional and functional characteristics [1–4]. Ternary receptors composed of two  $\alpha$ , two  $\beta$ , and one  $\gamma$  (or  $\delta$ ) subunits are considered to constitute the majority of GABA<sub>A</sub>Rs. On the other hand, the complexity of GABA<sub>A</sub>Rs lies in various allosteric modulatory sites [5,6]. Although knowledge concerning the GABA<sub>A</sub>R structure and its interaction with various ligands is increasing rapidly [5–11], a complete elucidation of the modulatory mechanisms of GABA<sub>A</sub>R is still far from being achieved.

As one of the most important GABA<sub>A</sub>R allosteric modulators, benzodiazepines (BZDs) have wide and versatile clinical applications. At low or high dosages, BZDs, such as

diazepam, produce anti-anxiety, anti-seizure, sedation, and anesthetic effects [12]. Conclusive evidence has shown that the anti-anxiety and sedative effects of BZDs are associated with the high-affinity (classical) sites located at the  $\alpha+$ / $\gamma-$  interface of synaptic GABA<sub>A</sub>Rs [13,14]. However, whether BZD-induced anesthesia is mediated by the classical binding sites remains to be clarified.

In addition to the classical binding sites, several new binding sites of BZDs on GABA<sub>A</sub>R are proposed [15–18]. In particular, a transmembrane BZD binding site that is activated by micromolar concentration of diazepam was suggested on recombinant  $\alpha_1\beta_2\gamma_2$  and  $\alpha_1\beta_2$  GABA<sub>A</sub>Rs. However, these in vitro experiments were conducted using limited BZDs and  $\alpha_1$ -containing GABA<sub>A</sub>Rs. Previously, we demonstrated the flumazenil-insensitive BZD effects in a series of  $\alpha\beta$  binary GABA<sub>A</sub>Rs [19] and in zebrafish larvae [20]. By using the electrophysiological technique, the present study revealed flumazenil-insensitive diazepam modulation on a series of ternary GABA<sub>A</sub>Rs, which was abolished by both the GABA<sub>A</sub>R antagonist pentylenetetrazol and Fa173, a proposed GABA<sub>A</sub>R antagonist targeting the transmembrane site [21]. The effects of structurally differentiated BZDs were further compared to verify the objectivity and universal significance of the non-classical site. Both pentylenetetrazol and Fa173, but not flumazenil, antagonized BZD-induced anesthesia, which is considered to be related to high-dose BZD effects. The present study provides novel evidence supporting the existence of a flumazenil-insensitive mechanism in BZD modulation of GABA<sub>A</sub>Rs.

## 2. Results

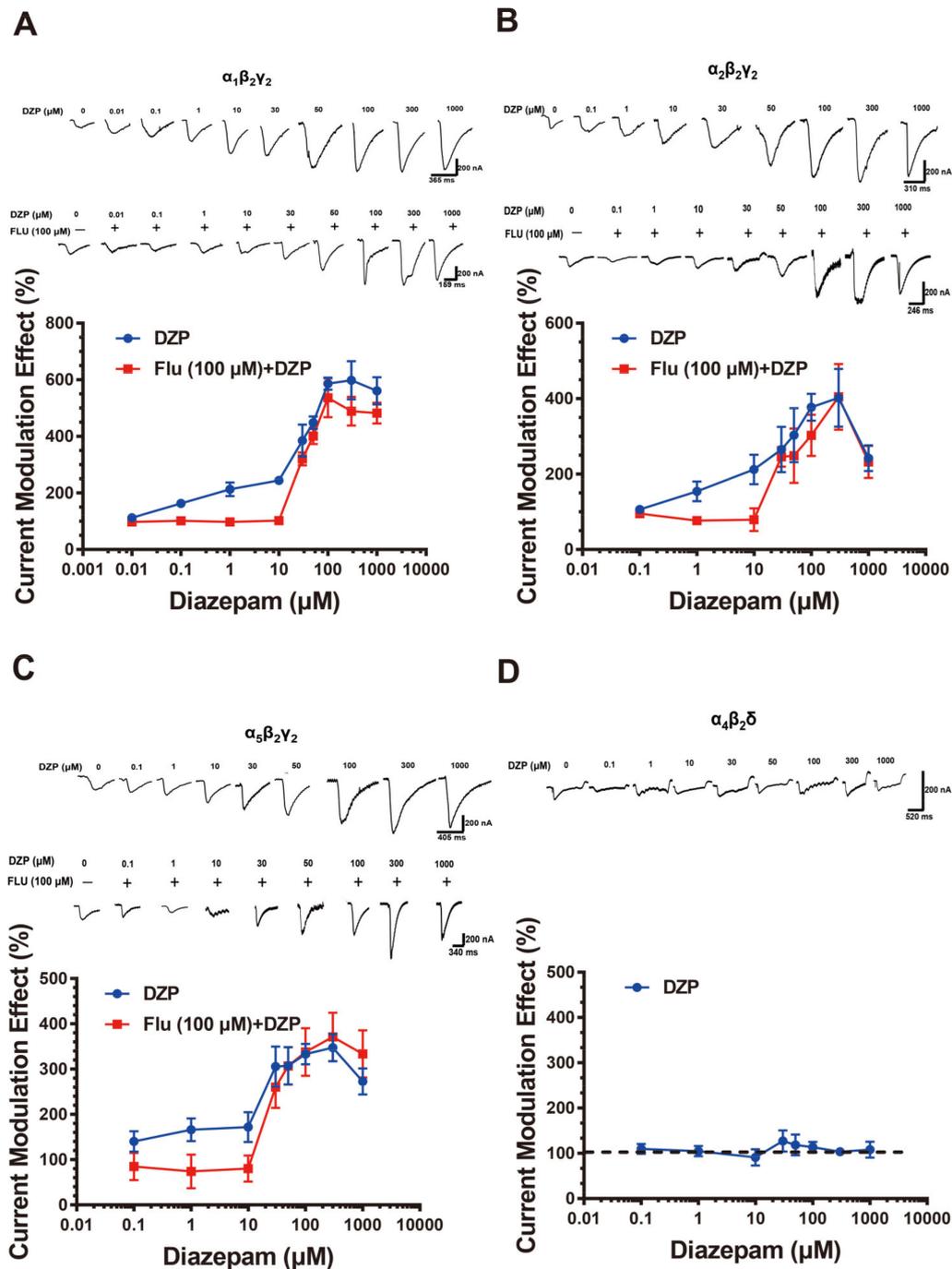
### 2.1. Diazepam Exhibits Flumazenil-Insensitive Modulation of $\alpha_1\beta_2\gamma_2$ , $\alpha_2\beta_2\gamma_2$ , and $\alpha_5\beta_2\gamma_2$ GABA<sub>A</sub>Rs

The effects of diazepam on the  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$ ,  $\alpha_5\beta_2\gamma_2$ , and  $\alpha_4\beta_2\delta$  receptors were observed across a broad range of concentrations (0.1 to 1000  $\mu$ M), in the presence of 1, 0.1, 1, and 0.1  $\mu$ M GABA respectively (these concentrations elicited 3~10% of the maximal GABA currents, EC<sub>3-10</sub>). Consistent with the previous study [15], diazepam potentiated the GABA-elicited currents on the  $\alpha_1\beta_2\gamma_2$  receptor in a biphasic manner (Figure 1A). The low- and high-concentration potentiation was at 0.1~10 and 10~1000  $\mu$ M respectively, and the latter could not be antagonized by the classical binding site antagonist flumazenil (even at a concentration as high as 100  $\mu$ M). Similarly, diazepam produced two components of potentiation in modulating the  $\alpha_2\beta_2\gamma_2$  and  $\alpha_5\beta_2\gamma_2$  receptors (Figure 1B,C). The first component of potentiation was induced by 0.1~10  $\mu$ M diazepam with a maximum of ~200% at both receptors. At concentrations above 10  $\mu$ M, diazepam evoked a second component of potentiation on the  $\alpha_2\beta_2\gamma_2$  and  $\alpha_5\beta_2\gamma_2$  receptors, further increasing GABA-elicited currents to approximately 400 and 350%, respectively. Flumazenil antagonized the action of diazepam at concentrations below 10  $\mu$ M, but not above. However, diazepam did not significantly affect the  $\alpha_4\beta_2\delta$  receptor (Figure 1D).

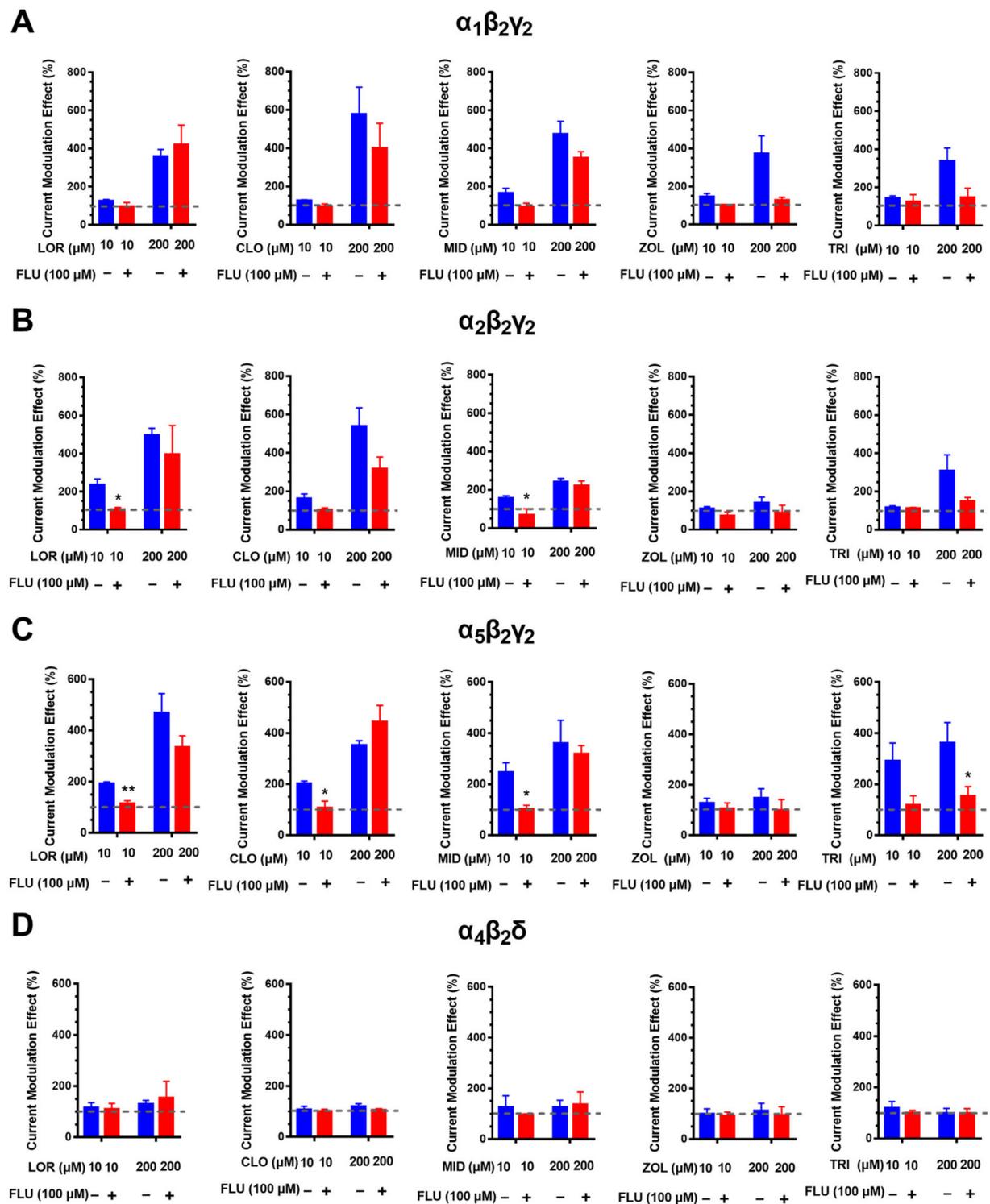
### 2.2. Different BZD Ligands Exhibit Distinct Flumazenil-Insensitive Modulation of Different GABA<sub>A</sub>Rs

Chemically differentiated BZD ligands, all of which bind to the classical  $\alpha/\gamma$  sites, were tested to clarify whether the high-dose potentiation was exclusive to diazepam. The concentrations of 10 and 200  $\mu$ M were selected to represent maxima of the low- and high-dose effects, respectively. Similar to diazepam, 10  $\mu$ M of lorazepam, clonazepam (both belonging to the 1,4-BZDs) and midazolam (imidazo-benzodiazepine) potentiated the GABA-elicited currents on the  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$  and  $\alpha_5\beta_2\gamma_2$  receptors, and these effects were abolished by flumazenil. The potentiation induced by 200  $\mu$ M lorazepam, clonazepam or midazolam was even greater on these three GABA<sub>A</sub>R subtypes, and these effects were resistant to flumazenil (Figure 2). Zolpidem (imidazopyridine), which was shown to be selective for the  $\alpha_1\beta_2\gamma_2$  receptor [22], produced a main and significant modulation on the  $\alpha_1\beta_2\gamma_2$  receptor, but its effects at both 10 and 200  $\mu$ M were antagonized by flumazenil. Triazolam (triazolo-benzodiazepine) positively modulated the  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$  and  $\alpha_5\beta_2\gamma_2$  receptors, but its effects at both 10 and 200  $\mu$ M were also substantially antagonized by

flumazenil. In addition, all of the ligands tested exhibited limited modulatory effects on the  $\alpha_4\beta_2\delta$  receptor, at either 10 or 200  $\mu\text{M}$ .



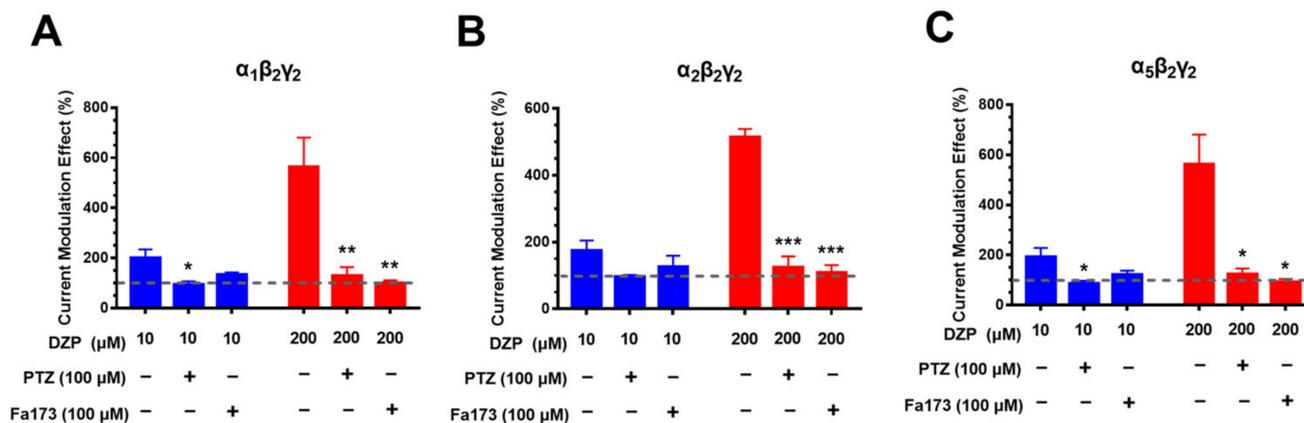
**Figure 1.** Diazepam modulated the  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$  and  $\alpha_5\beta_2\gamma_2$  receptors in flumazenil-sensitive and flumazenil-insensitive manners. The concentration-response relationships of diazepam for modulating the  $\alpha_1\beta_2\gamma_2$  (A),  $\alpha_2\beta_2\gamma_2$  (B),  $\alpha_5\beta_2\gamma_2$  (C), and  $\alpha_4\beta_2\delta$  (D) receptors were determined in the absence or presence of flumazenil. The GABA concentrations were 1, 0.1, 1, and 0.1  $\mu\text{M}$  for  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$ ,  $\alpha_5\beta_2\gamma_2$ , and  $\alpha_4\beta_2\delta$  receptors, respectively. Representative current traces are shown on the top; data summary is shown on the bottom. Data represent mean  $\pm$  SEM,  $n = 4-5$ .



**Figure 2.** Different BZD ligands exhibited distinct modulation of ternary GABA<sub>A</sub>Rs. Effects of lorazepam (LOR), clonazepam (CLO), midazolam (MID), zolpidem (ZOL) and triazolam (TRI) in potentiating the GABA-elicited currents were evaluated at low (10  $\mu\text{M}$ ) and high (200  $\mu\text{M}$ ) concentrations in the absence or presence of flumazenil (100  $\mu\text{M}$ ) on the  $\alpha_1\beta_2\gamma_2$  (A),  $\alpha_2\beta_2\gamma_2$  (B),  $\alpha_5\beta_2\gamma_2$  (C) and  $\alpha_4\beta_2\delta$  (D) receptors. The dotted lines indicate the basal levels without drug treatment. Data represent mean  $\pm$  SEM,  $n = 4$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , vs control group, via  $t$ -test.

### 2.3. Pentylentetrazol and Fa173 Abolish the Flumazenil-Insensitive Diazepam Effects

To exclude the possibility that the high-concentration BZD effects were a type of nonspecific effect, the effects of 200  $\mu\text{M}$  diazepam were observed in the presence of either the GABA<sub>A</sub>R antagonist pentylentetrazol or Fa173, a proposed transmembrane site antagonist [21]. Similarly, on the  $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$  and  $\alpha_5\beta_2\gamma_2$  receptors, diazepam produced two-graded potentiation on the GABA-elicited current at 10 and 200  $\mu\text{M}$ . 100  $\mu\text{M}$  PTZ or Fa173 antagonized not only the low-concentration but also the high-concentration effects of diazepam (Figure 3). These results indicated that the flumazenil-insensitive effects are specifically mediated by GABA<sub>A</sub>Rs, and possibly via the transmembrane binding sites.



**Figure 3.** Pentylentetrazol and Fa173 abolished the flumazenil-insensitive diazepam effects on ternary GABA<sub>A</sub>Rs. Effects of diazepam (DZP) in potentiating the GABA-elicited currents were evaluated at low (10  $\mu\text{M}$ ) and high (200  $\mu\text{M}$ ) concentrations in the absence or presence of pentylentetrazol (PTZ, 100  $\mu\text{M}$ ) or Fa173 (100  $\mu\text{M}$ ) on the  $\alpha_1\beta_2\gamma_2$  (A),  $\alpha_2\beta_2\gamma_2$  (B) and  $\alpha_5\beta_2\gamma_2$  (C) receptors. The dotted lines indicate the basal levels without drug treatment. Data represent mean  $\pm$  SEM,  $n = 4$ , \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs. control group, via  $t$ -test.

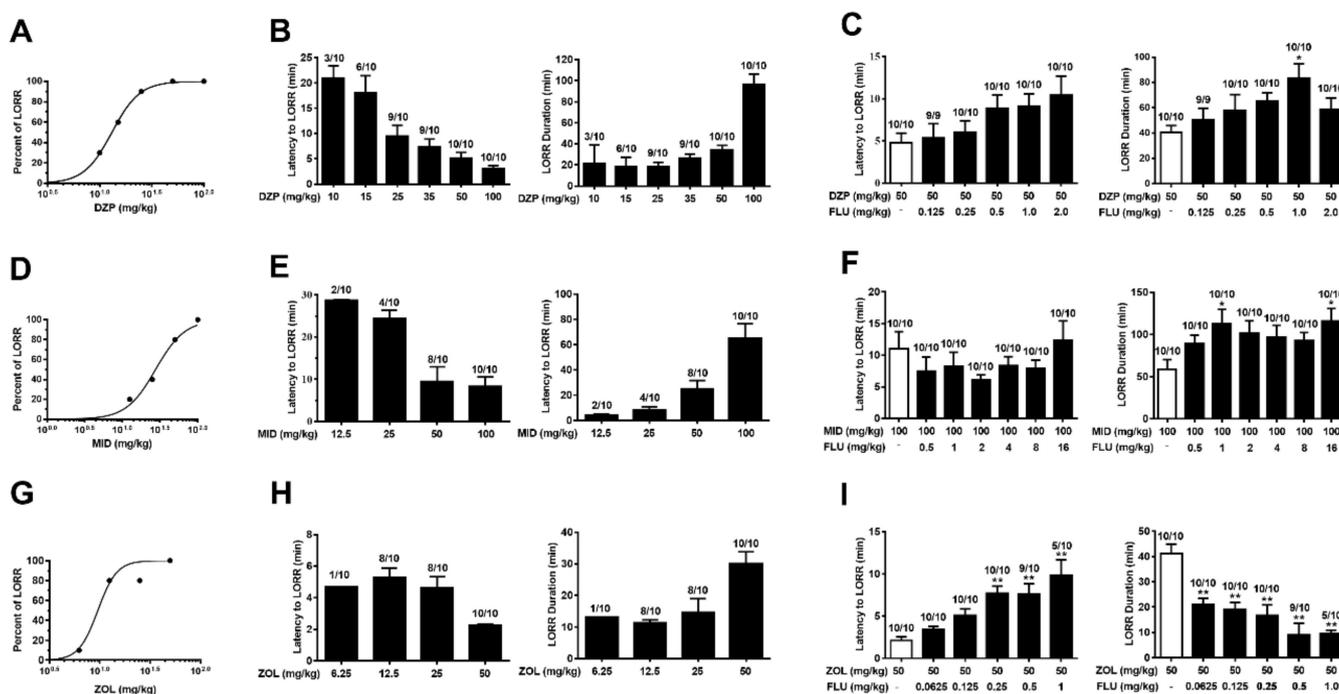
### 2.4. Anesthesia Induced by Diazepam and Midazolam, but Not Zolpidem, Is Resistant to Flumazenil

The high-dose effects of BZD ligands were evaluated *in vivo* by using LORR as an index of anesthesia. Diazepam, midazolam and zolpidem were chosen to compare the potential flumazenil-insensitive effects of different ligands. All three ligands dose-dependently induced LORR in mice (Figure 4A,D,G), and an increase in BZD dose led to a decrease in latency to and an increase in duration of LORR. Complete (100%) LORR was caused by diazepam, midazolam and zolpidem at doses of 50, 100 and 50  $\text{mg kg}^{-1}$ , respectively. Flumazenil treatment failed to antagonize LORR induced by diazepam or midazolam. Moreover, flumazenil even prolonged the duration of LORR at some doses (Figure 4C,F). In contrast, zolpidem-induced anesthesia was significantly antagonized by flumazenil. Flumazenil dose dependently increased the latency ( $F(5, 53) = 9.82$ ,  $p < 0.01$ ) and reduced the duration ( $F(5, 53) = 11.06$ ,  $p < 0.01$ ) of LORR induced by zolpidem. In addition, the percentage of zolpidem-induced LORR was reduced to 50% by flumazenil at a dose of 1  $\text{mg kg}^{-1}$  (Figure 4I).

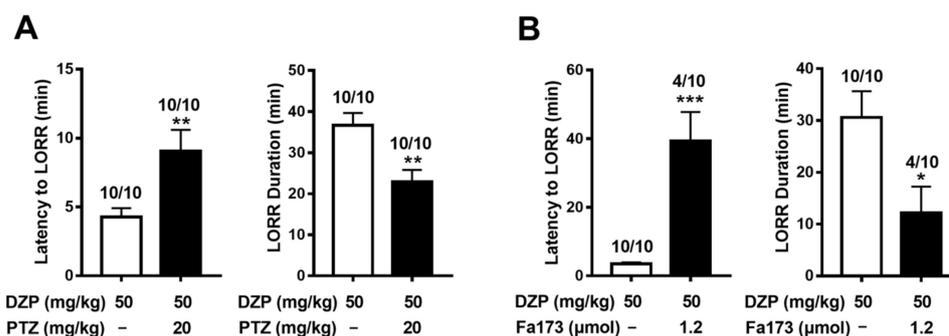
### 2.5. Diazepam-Induced Anesthesia Is Antagonized by Pentylentetrazol and Fa173

Pentylentetrazol and Fa173 were further used to determine the specific target for the high-dose BZD effects in living animals. Compared with the control, pentylentetrazol treatment significantly increased the latency to ( $t = 2.88$ ,  $p < 0.01$ , Figure 5A) and reduced the duration ( $t = 3.3$ ,  $p < 0.01$ ) of LORR induced by diazepam, indicating that the high-dose BZD effects were mediated via GABA<sub>A</sub>Rs. Similarly, Fa173 treatment antagonized diazepam-induced LORR, resulting in significant decreases in LORR percentage ( $p < 0.05$ , Figure 5B) and duration ( $t = 2.571$ ,  $p < 0.05$ ), and an increase in LORR latency ( $t = 4.247$ ,  $p <$

0.001). This was consistent with the in vitro results of Fa173 and suggested that high-dose diazepam activates the transmembrane binding sites of GABA<sub>A</sub>Rs.



**Figure 4.** Flumazenil antagonized LORR induced by zolpidem, but not by diazepam or midazolam. A series of doses of diazepam (DZP), midazolam (MID) and zolpidem (ZOL) were administered intraperitoneally, and percentage of LORR (A,D,G) and latency to and duration of LORR (B,E,H) were recorded. Flumazenil (FLU) was intravenously injected immediately before BZD administration, and its effects on LORR induced by 50 mg kg<sup>-1</sup> diazepam, 100 mg kg<sup>-1</sup> midazolam or 50 mg kg<sup>-1</sup> zolpidem were evaluated (C,F,I). Data represent mean  $\pm$  SEM,  $n = 9-10$ . Figure on top of each bar is the number of mice that lost the righting reflex over the total number of the mice tested. One-way ANOVA; \*  $p < 0.05$ , \*\*  $p < 0.01$  vs control group, according to post hoc analysis with Dunnett's multiple comparison test.



**Figure 5.** Pentylentetrazol and Fa173 antagonized diazepam-induced LORR. Pentylentetrazol (PTZ, 20 mg kg<sup>-1</sup>, i.v.) or Fa173 (1.2  $\mu$ mol/mouse, i.c.v.) was injected immediately before diazepam (DZP, 50 mg kg<sup>-1</sup>, i.p.) administration. The effect of pentylentetrazol (A) or Fa173 (B) on latency to and duration of diazepam-induced LORR were recorded. Data represent mean  $\pm$  SEM,  $n = 10$ . Figure on top of each bar is the number of mice that lost the righting reflex over the total number of the mice tested. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  vs control group, via  $t$ -test.

### 3. Discussion

The present study demonstrated that high doses of BZDs produced profound and flumazenil-insensitive potentiation of GABA-elicited currents on certain GABA<sub>A</sub>R subtypes. Consistent with the electrophysiological observations, the anesthesia induced by diazepam, the BZD phenotype, was resistant to flumazenil, but antagonized by pentylentetrazol or Fa173. The findings of this study support the existence of a non-classical mechanism in GABA<sub>A</sub>R modulation that may contribute to BZD-induced anesthesia.

Direct experimental evidence is still lacking concerning BZD concentration around synaptic and extra-synaptic GABA<sub>A</sub>Rs after systemic administration, although it is important to determine the clinical significance of high-dose BZD effects. Based on the measured plasmic levels and their high lipid solubility, the concentrations of BZDs in the CNS were estimated to reach double-digit micromolar levels [23,24]. It is reasonable to speculate even higher levels of BZDs in specific brain regions and neural circuits under some circumstances of iatrogenic or self-inflicted overdose. Therefore, the local BZD concentrations were very likely to be high enough for flumazenil-insensitive potentiation under some practical conditions, especially in terms of BZD-induced anesthesia and intoxication.

In contrast to the extensive research on the high-affinity modulatory effects of BZD at nanomolar concentrations, very limited studies have addressed the effects and mechanisms of micromolar BZD. Moreover, there are controversial observations regarding the interaction of low- and high-affinity BZD effects. High-concentration flurazepam inhibited the GABA<sub>A</sub>R modulation mediated by the classical BZD binding site [15,25], while diazepam at concentrations above 20 μM further potentiated the α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptor [15]. Our results are in good agreement with the latter, and extend the finding on diazepam and the α<sub>1</sub>β<sub>2</sub>γ<sub>2</sub> receptor to a series of ligands and receptors. The flumazenil-insensitive high-dose effects of classical BZDs on synaptic GABA<sub>A</sub>Rs support the existence of non-classical binding sites, precluding the possibility that the micromolar potentiation of diazepam is a non-specific effect. The fact that Fa173 abolishes the high-dose BZD effects further verifies the non-classical binding sites, possibly located at the transmembrane domain (TMD) of GABA<sub>A</sub>Rs. However, other non-classical mechanisms may contribute to high-dose BZD effects [26].

The high-dose BZD effects may mean a broader and deeper depression of the central nervous system, which is considered to be related to general anesthesia [27]. In good agreement with previous studies [20], the full occupation of the non-classical binding sites by high concentrations of BZDs resulted in 2–3-fold increases in the maximum effects relative to modulation via the classical binding sites. Furthermore, the high-dose BZD effects are assumed to affect more GABA<sub>A</sub>R subtypes, as the construction of the non-classical binding sites does not require the γ subunit [19]. It is also interesting to see that the non-classical binding sites possessed similar dependence on the α subunit compared to the classical binding sites; i.e., α<sub>1</sub>-, α<sub>2</sub>- or α<sub>5</sub>-, but not α<sub>4</sub>-containing receptors were sensitive to BZD modulation. On the other hand, high-dose modulation was observed in classical BZDs but not non-BZD structures (such as zolpidem), although both categories of ligands bind to the classical binding sites of GABA<sub>A</sub>Rs. Similarly, a recent study has suggested that the structural features of BZD ligands govern their abilities to bind to the etomidate binding site of the GABA<sub>A</sub>Rs [28].

Flumazenil, which competes with BZDs to bind to the classical binding site, is the only specific therapeutic for BZD intoxication in clinic. Extensive evidence supports the effective antagonism of flumazenil on sedation, anti-anxiety, and anti-convulsion activity induced by relatively low doses of diazepam [29–31], while its effects on high-dose BZDs are rarely studied. The present study investigated the effectiveness of flumazenil against BZD-induced anesthesia, using the LORR as an *in vivo* model representing high-dose BZD effects. Flumazenil failed to effectively antagonize LORR induced by diazepam and midazolam. In particular, LORR duration was not reduced, but even prolonged by flumazenil under some doses. This result, which was consistent with the *in vitro* results, suggests that there is a flumazenil-insensitive mechanism in BZD-induced modulation of

GABA<sub>A</sub>Rs, and that flumazenil may be inefficient in antagonizing some BZD effects, such as anesthesia, when used against high doses of certain BZDs.

The binding modes and mechanistic effects of BZD ligands were increasingly resolved, as the high-resolution structures of GABA<sub>A</sub>Rs in complex with BZDs were presented. Strong densities were observed in the TMD of the  $\alpha_1\beta_3\gamma_2$  receptor, which is also considered the binding site for general anesthetics [9]. Recently, diazepam was demonstrated to share a binding site with anesthetics in the TMD of the  $\alpha_1\beta_2\gamma_2$  receptor [11]. The binding of diazepam to this site may contribute to stabilization of the TMD and positively modulate the receptor in a similar way to anesthetics. These structural studies of GABA<sub>A</sub>Rs provided an excellent explanation for the flumazenil-insensitive BZD effects and the non-classical BZD modulatory mechanism.

In conclusion, the present study provided detailed evidence supporting the existence of flumazenil-insensitive BZD effects in a series of GABA<sub>A</sub>Rs and their potential association with BZD-induced anesthesia. These findings enhance the understanding of GABA<sub>A</sub>R modulation and BZD pharmacology, and suggest that some classical BZDs produce profound inhibition of brain function by binding to non-classical sites. Furthermore, elaborate analysis of the interaction of ligands with non-classical sites may prompt the development of novel drugs in modulating wakefulness and sleep [21,32].

## 4. Materials and Methods

### 4.1. Chemicals

GABA was obtained from Sigma-Aldrich (St. Louis, MO, USA). Flumazenil (PubChem ID: 3373), clonazepam (PubChem ID: 2802), lorazepam (PubChem ID: 3985), zolpidem (PubChem ID: 5732), triazolam (PubChem ID: 5556) were purchased from the National Institutes for Food and Drug Control (Beijing, China). Diazepam (PubChem ID: 3016), midazolam (PubChem ID: 4192), pentylentetrazol (PubChem ID: 5917) were obtained from Jiangsu Enhua Pharmaceutical Co., Ltd. (Xuzhou, China). Fa173 was synthesized and provided by Beijing ShiKang Synthesis Pharmaceutical Co., Ltd. All drugs were dissolved in DMSO and stored at  $-20\text{ }^{\circ}\text{C}$  and were diluted to the needed concentrations using recording solution at room temperature on the experimental day.

### 4.2. Expression of GABA<sub>A</sub> Receptors in *Xenopus* Oocytes

The subcloning of human  $\alpha_1$ ,  $\alpha_2$ ,  $\alpha_4$ ,  $\alpha_5$ ,  $\beta_2$ ,  $\gamma_2$ , and  $\delta$  cDNAs into the pGH19 vector was performed as described previously [33]. The cRNA was transcribed from the linearized cDNAs via standard in vitro transcription procedures using the T7 mMESSAGE mMACHINE High Yield Capped RNA transcription kit (Invitrogen). Oocytes were harvested from anesthetized adult female *Xenopus laevis*, dispersed and incubated in oocyte Ringer (OR<sub>2</sub>, 82 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1 mM MgCl<sub>2</sub>, pH 7.6) plus 0.8 mg/mL collagenase A (Sigma, USA) for approximately 1 h. After isolation, the oocytes were thoroughly rinsed with OR<sub>2</sub> and stage V or VI oocytes were separated and selected. On the next day, oocytes were injected with 40 ng of cRNA mixtures encoding  $\alpha_{1/2/5}\beta_2\gamma_2$  and  $\alpha_4\beta_2\delta$  at a ratio of 1 $\alpha$ :1 $\beta$ :1 $\gamma$ / $\delta$ . Injected oocytes were incubated at 18 °C in ND96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.6). The oocytes expressing synaptic ( $\alpha_1\beta_2\gamma_2$ ,  $\alpha_2\beta_2\gamma_2$ ,  $\alpha_5\beta_2\gamma_2$ ) and extra-synaptic ( $\alpha_4\beta_2\delta$ ) GABA<sub>A</sub>Rs were used for electrophysiological recording 1–3 and 4–6 days after cRNA injection, respectively.

### 4.3. Two-Electrode Voltage Clamp Electrophysiology

Whole-cell currents were measured using the two-electrode voltage clamp technique. Microelectrodes were filled with 3 M KCl and those with resistance between 1.0–2.5 M $\Omega$  were used. Recordings were performed under constant perfusion at room temperature. Currents were amplified with an OC-725C (Warner Instruments, Hamden, USA) and digitized with a Digidata 1440 (Molecular Devices, San Jose, CA, USA) at 100 Hz. In all cases, currents in response to the application of drugs were recorded using Clampex 10.3 software (Axon Instruments, San Jose, CA, USA) and data were sampled at 2 kHz and

filtered at 0.5 kHz. A gap-free protocol was applied with the holding membrane potential at  $-70$  mV. Each drug application was followed by a washout in bath solution (approximately 5 min).

#### 4.4. Mouse Behavioral Test

Adult male Kunming mice ages 3–4 weeks (19–21 g) were obtained from Beijing Animal Center (Beijing, China). Animals were housed 10 per cage with free access to food and water on a 12 h light–dark cycle. The mice were handled for 2–3 days to adapt to experimental conditions. All experimental procedures were approved by the local ethical committee and the Institutional Review Committee on Animal Care and Use (IACUC of AMMS-06-2017-003).

The anesthetic effects of BZDs were measured using a mouse LORR model. The mice were tested individually in a clear plastic cage ( $40 \times 20 \times 20$  cm,  $l \times w \times h$ ). After BZD injection, the mice were gently placed in the supine position and the righting reflex was assessed every 1 min until the occurrence of LORR, which manifested as the failure to right themselves within 60 s. The anesthetized mice were left undisturbed until they spontaneously turned over themselves to prone position. Absolute recovery was defined as the mice being able to right themselves twice or more within 60 s. The time between BZD injection and occurrence of LORR was recorded as latency to LORR, and LORR duration was measured as the time from the occurrence of LORR to recovery. The LORR was considered absent if the mice were able to right themselves during the 120 min observation period after BZD injection.

#### 4.5. Statistical Analysis

Electrophysiological data were analyzed with Clampfit 10.3 (Axon Instruments, San Jose, USA), Origin 8.0 (OriginLab Corporation, Northampton, MA, USA) and GraphPad Prism 5.0 (GraphPad Software Inc, La Jolla, CA, USA). Responses were normalized to the maximal response elicited by GABA. All data were presented as mean  $\pm$  SEM, comparisons between groups were analyzed using one-way analysis of variance (ANOVA) with Dunnett's post hoc test or unpaired *t*-test, and significant differences were considered if  $p < 0.05$ .

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