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Flumazenil-insensitive benzodiazepine binding sites in GABA$_A$ receptors contribute to benzodiazepine-induced immobility in zebrafish larvae

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ABSTRACT

Aims: Benzodiazepines (BZDs) produce various pharmacological actions by binding to and allosterically regulating GABA$_A$ receptors. Several *in vitro* studies have demonstrated diazepam, the prototypic BZD, produces a high-dose action that cannot be countered with the classical BZD-binding site antagonist flumazenil. Here, we investigate the existence and behavioral relevance of non-classical BZD binding sites in zebrafish larvae.

Main methods: Zebrafish larvae were treated with a series of BZDs alone or combined with flumazenil, bicuculline (a non-selective GABA$_A$ receptor antagonist), or RO 15–4513 (a general BZD antagonist and a proposed antagonist interacting with $\alpha+/\beta-$ interfaces in $\alpha_{4/6}/\beta_3\delta$ receptors), and their locomotor activities and behavioral phenotypes were recorded.

Key findings: Diazepam-induced hypolocomotion (sedation-like state) at low doses (10 and 20 $\mathrm{mg\,L^{-1}}$) was effectively antagonized by flumazenil or bicuculline, while diazepam-induced immobility (anesthesia-like state) at higher dose (30 $\mathrm{mg\,L^{-1}}$) was prevented by bicuculline (3 $\mathrm{mg\,L^{-1}}$) but not flumazenil, even at doses up to 150 $\mathrm{mg\,L^{-1}}$. Ro 15–4513 also failed to efficiently antagonize diazepam-induced immobility. Immobility induced by high dose of another 1,4-BZD, clonazepam, was also resistant to flumazenil.

Significance: These results provide direct *in vivo* evidence for non-classical BZD-binding sites, which may be located at the second transmembrane domain of GABA$_A$ receptors and contribute to BZD-induced anesthesia.

1. Introduction

γ-Aminobutyric acid type A receptors (GABA$_A$Rs), which are the major inhibitory transmitter receptors in the mammalian central nervous system, are natively composed of a pentameric arrangement of 19 possible subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, ρ_{1-3}, and θ) [1,2]. GABA$_A$Rs at synaptic sites mediating fast inhibitory postsynaptic potentials are predominantly composed of $\alpha_{1/2/3/5}$, β_x, and γ_2 subunits; while those at extrasynaptic sites mediate tonic inhibition and comprise $\alpha_{4/5/6}$, β_x, and δ subunits [3,4]. Studies of GABA$_A$Rs using mutagenesis, photolabeling, and docking methods have identified several binding sites for numerous sedative/anesthetic drugs such as benzodiazepines (BZDs), barbiturates and intravenous anesthetics [3,5,6]. However, the allosteric regulation of GABA$_A$Rs and pharmacological mechanisms of these drug binding sites still needs elaboration, especially at *in vivo* levels.

Benzodiazepines (BZDs) are widely used as anxiolytics, sedatives, and anesthetics in the clinic [7,8]. Classical theories suggest that BZDs produce anti-anxiety and sedative effects by binding to the high-affinity sites located at the $\alpha_{1/2/3/5}+/\gamma_2-$ interface of synaptic GABA$_A$Rs to enhance GABA-mediated hyperpolarization of the postsynaptic membrane [3,5,6,8]. However, it is unclear whether these classical sites are fully responsible for BZD-induced anesthesia. A previous study in *Xenopus* oocytes demonstrated that the recombinant $\alpha_1\beta_2\gamma_2$ subtype receptor contains another BZD-binding site that is insensitive to the classical BZD antagonist flumazenil [9]. Binding of diazepam to this non-classical site produces a second concentration-dependent potentiation of GABA-elicited currents. Further studies on neocortical slices reported that diazepam at higher concentrations evokes a stronger depression of action potential firing, which is resistant to flumazenil but is reduced by the non-selective GABA$_A$R antagonist bicuculline [10]. Therefore, additional non-classical BZD-binding sites in GABA$_A$Rs may be involved in the high-dose action of diazepam.

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However, there is insufficient evidence for the existence of these sites *in vivo*, and their behavioral relevance is unclear.

Zebrafish (*Danio rerio*), a diurnal vertebrate, is a powerful model for neuropharmacological studies due to its high homology in the genome, nervous system, and behavioral phenotype compared to mammals [11–13]. This species possesses a well-developed GABAergic signaling system and high sensitivity to GABAergic sedatives [14,15]. Zebrafish can absorb environmental agents through their skin and gills. This feature makes it feasible and convenient to evaluate the behavioral consequences of drugs at constant levels by adding them directly into the tank water [16]. In this study, we used zebrafish instead of traditional experimental animals to investigate the effects of BZDs alone or in combination with antagonists without concentration variation, and thus to exclude the pharmacokinetic factors that may complicate the explanation of drug interactions [17,18].

Here, we investigated the detailed dose-dependent effects of diazepam and other BZDs on zebrafish larvae behaviors, with a focus on their high-dose actions. We compared the antagonism of flumazenil, bicuculline, and Ro 15–4513 (a general BZD antagonist and a proposed antagonist interacting with $\alpha+/\beta-$ interfaces in $\alpha_{4/6}/\beta_3\delta$ receptors [19]) on the effects of BZDs to verify the existence, receptor subtype localization, and pharmacological characteristics of non-classical BZD-binding sites, which may be related to BZD-induced anesthesia *in vivo*.

2. Materials & methods

2.1. Zebrafish maintenance and embryo handling

Adult male and female zebrafish (AB strain, 3–6 months old) were maintained in a breeding tank at a 1:1 ratio and kept on a 12-h light/dark cycle (light on at 8:00 a.m. and off at 8:00 p.m.). Adult fish were fed with frozen brine shrimp every 12 h. Naturally spawned embryos were raised in groups of 80 in a 10 cm cell culture dish (Corning, New York, USA) filled with 20 mL E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.4). Embryos and larvae were kept in a 28.5 °C incubator. Every 24 h, the medium was replaced, and unhatched embryos were removed. All experiments were approved by the Ethics Committee and the Institutional Animal Care and Use Committee of Beijing Institute of Pharmacology and Toxicology, Beijing, China (IACUC of AMMS-06-2016-012) and were conducted in accordance with their guidelines.

2.2. Compounds and drugs

Diazepam, flumazenil, clonazepam, lorazepam, triazolam, midazolam, and zolpidem were obtained from the Chinese National Institutes for Food and Drug Control (Beijing, China). Bicuculline and Ro 15–4513 were purchased from Tocris Bioscience (Bristol, UK). Stock solutions of all drugs were prepared in dimethyl sulfoxide (DMSO) and diluted with E3 embryo medium on the day of experimentation. The final DMSO concentration of all drugs and control solutions was 5% and did not affect the basal locomotor activity of zebrafish larvae at 6 days post-fertilization (dpf) with treatment duration of 30 min [20,21].

2.3. Locomotor activity measurement and quantification

The behavioral tests were performed with zebrafish larvae (6 dpf) between 8:30 a.m. and 12:00 a.m. The larvae were first placed into a single well of a 12-well plate, which was loaded with a 15 mm Netwell Insert (Corning, New York, USA) in each well. Subsequently, the Netwell inserts were transferred into another 12-well plate in which each well contained 5 mL E3 embryo medium. To study the antagonism of bicuculline on diazepam-induced motor inhibition, the larvae were first incubated with bicuculline for 10 min (as bicuculline has a slow onset of action) and then transferred into the mixed solution of diazepam and corresponding bicuculline. Otherwise, the larvae were treated with BZDs alone or in combination with flumazenil or Ro 15–4513. The doses of these antagonists were chosen following preliminary experiments to effectively antagonize BZDs at different doses. Then, the larvae were carefully transferred into a 48-well plate with a single larva in each well. The volume of the drug solution in each well was maintained at 300 μL to ensure adequate swimming space and prevent any vertical movement of the larvae. Subsequently, the 48-well plate was placed into a Zebrabox apparatus (Viewpoint Life Sciences Inc., Lyon, France) to record the locomotor activity of zebrafish larvae for 30 min. At the end of the experiments, the wells were examined using a dissecting microscope and those containing deformed larvae or more than one larva were excluded from the analysis.

Videos recorded during testing were real-time quantified using the tracking mode of the Zebralab software (Viewpoint Life Sciences Inc.). The videos were taken at a rate of 25 frames per second. The Videotrack parameters were set as follows: detection threshold, 9; burst (threshold for very large movement), 20; freeze (threshold for no movement), 1; bin size, 5 min. The distance of larval movement in the well at each stage was acquired for the analysis of locomotor activity.

2.4. Behavioral phenotype determination

The status of the zebrafish larvae was defined based on their behavioral manifestation during the last 5 min (26–30 min) after drug treatment, referring to a previous report [22]. As the vehicle-treated larvae swam 701.9 ± 170.9 mm (mean ± standard deviation, $n = 96$) in this 5-min period, the larvae were considered to enter a sedative-like state if their swimming distance was less than 189 mm, according to the three-sigma rule in statistics. The immobility of larvae (no actual movement) was judged by a combination of automated tracking quantification and individual inspection by a trained experimenter blinded to the treatments.

2.5. Lethal action of drugs

Zebrafish larvae were randomly divided into groups of 10 and placed in one well of the 12-well plate. The Netwell inserts with the larvae were immersed for 1 h in a new 12-well plate in which each well contained a different drug dose. The drug solutions in each well were replaced with the medium three times to remove any residual drugs, and the larvae were kept in the medium. The numbers of dead larvae were recorded 24 h later.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). To determine the time-course of the effect of different treatments on larval locomotor activities, the swimming distance at each 5-min bin was analyzed using two-way repeated measures analysis of variance (ANOVA) with time as the repeated variable. The overall locomotor activity was calculated as the percentage of the total swimming distance during the 30-min observation relative to that of the control group, which followed the same experimental procedures but received vehicle treatment, and was compared using a one-way ANOVA. A Bonferroni post hoc test was then employed to examine the differences among the groups. The antagonism efficiency of flumazenil on diazepam-induced motor inhibition was fitted using the following equation: Antagonism efficiency (%) = $(D_{DZP+FLU} - D_{DZP})/(D_{control} - D_{DZP}) \times 100\%$, where D, DZP, and FLU are distance, diazepam, and flumazenil, respectively. Non-linear regression was used to fit the dose-response curve and calculate the half maximal efficacy dose (ED_{50}) or median lethal dose (LD_{50}). The numbers of zebrafish larvae kept in normal, sedative, and immobile states in the control and drug-treated groups were recorded and analyzed using the Chi-square test. For all analyses, differences with $p < 0.05$ were considered significant.

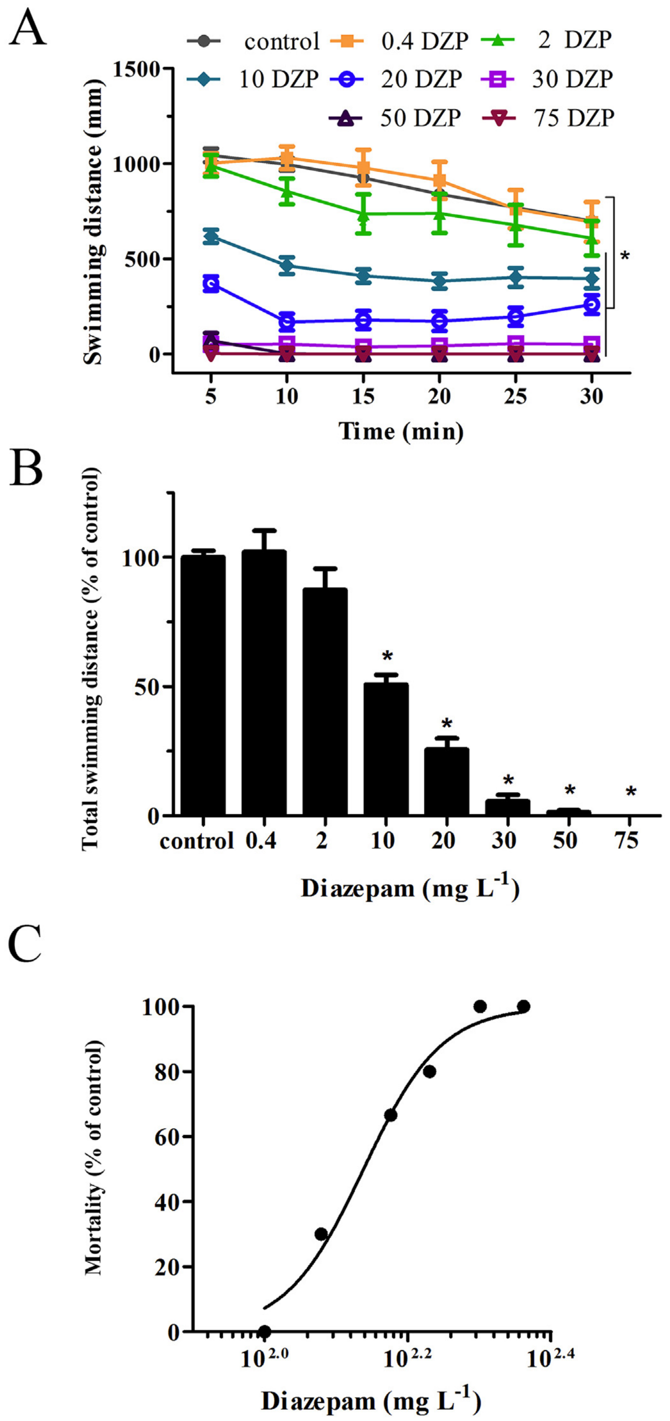

Fig. 1. Effects of diazepam on locomotor activity and survival in zebrafish larvae. (A) Swimming distance of zebrafish larvae treated with diazepam at doses of 0.4, 2, 10, 20, 30, 50, and 75 mg L^{-1} in each 5-min bin. (B) Percentages of total swimming distance of zebrafish larvae in the diazepam-treated groups relative to the control group (ED$_{50}$ = 9.91 mg L^{-1}). (C) Dose-response curve of the lethal effect of diazepam on zebrafish larvae (LD$_{50}$ = 137.4 mg L^{-1}). *indicates significantly different from the control group ($p < 0.05$). DZP, diazepam.

Table 1
Behavioral characteristics of zebrafish larvae after benzodiazepine treatment.

Benzodiazepine	Dose mg L^{-1}	Behavioral characteristics (%)			p value
		Normal	Sedation-like	Anesthesia-like	
Diazepam	vehicle	100.0	0.0	0.0	–
	10	68.8	31.2	0.0	ns
	20	45.5	40.9	13.6	< 0.001
	30	12.5	25.0	62.5	< 0.001
	50	0.0	0.0	100.0	< 0.001
	75	0.0	0.0	100.0	< 0.001
Clonazepam	vehicle	100.0	0.0	0.0	–
	10	87.5	12.5	0.0	ns
	50	0.0	62.5	37.5	< 0.001
	100	0.0	0.0	100.0	< 0.001
Lorazepam	10	12.5	87.5	0.0	ns
	50	0.0	85.7	14.3	< 0.001
	100	0.0	62.5	37.5	< 0.001
Trizolam	10	37.5	62.5	0.0	< 0.01
	50	50.0	25.0	25.0	ns
	100	66.7	33.3	0.0	ns
Zolpidem	10	100.0	0.0	0.0	ns
	50	100.0	0.0	0.0	ns
	100	100.0	0.0	0.0	ns
Midazolam	vehicle	100.0	0.0	0.0	–
	5	56.3	25.0	18.7	< 0.05
	10	0.0	14.3	85.7	< 0.001
	15	0.0	0.0	100.0	< 0.001

Different doses of benzodiazepines were added to the tank water, and larval behaviors were recorded during the 26–30 min after treatment. The definitions of normal, sedative, and immobile behavioral phenotypes are described in detail in the METHODS section. The differences in larvae numbers in three states between the drug-treated groups and the control group were compared using the Chi-square test. n = 8–40. The level of statistical significance was defined as a p value less than 0.05. ns, not significantly different.

3. Results

3.1. Diazepam dose-dependently decreased the locomotor activity of zebrafish larvae

A detailed investigation of the effect of diazepam on the swimming distance of zebrafish larvae was performed at doses of 0.4, 2, 10, 20, 30, 50, and 75 mg L^{-1} in each 5-min time bin (Fig. 1A). A two-way repeated measures ANOVA demonstrated a significant influence of treatment ($F_{7,855} = 113.5$, $p < 0.0001$), time ($F_{5,855} = 32.97$, $p < 0.0001$), and a treatment × time interaction ($F_{35,855} = 5.528$, $p < 0.0001$). A Bonferroni post hoc comparison indicated that diazepam at 0.4 mg L^{-1} (1.4 μM) did not affect the larval activity, consistent with a previous study [23]. When diazepam doses were above 2 mg L^{-1}, the larval activities were inhibited at least at one time point. Diazepam dose-dependently decreased the total locomotor activity in the 30-min observation period with an ED$_{50}$ of 9.91 mg L^{-1} (Fig. 1B).

At doses above 30 mg L^{-1}, diazepam produced dramatic inhibition of larval activities (Fig. 1A). The percentages of zebrafish larvae kept immobile in the 30, 50, and 75 mg L^{-1} diazepam-treated groups during the last 5 min were 62.5%, 100%, and 100% respectively (Table 1). By contrast, the larvae in the 10 and 20 mg L^{-1} diazepam-treated groups still retained low-level locomotor activity (Fig. 1A, Table 1). In another group of zebrafish larvae, the lethality induced by high-dose diazepam was evaluated (Fig. 1C). The LD$_{50}$ of diazepam was 137.4 mg L^{-1}, and diazepam at doses less than 100 mg L^{-1} had no lethal action.

3.2. Diazepam-induced immobility is resistant to flumazenil

The antagonism of flumazenil on diazepam-induced motor inhibition at 10, 20, and 30 mg L^{-1} was further observed (Fig. 2 A–C, left). A one-way ANOVA revealed a significant difference among the total locomotor activity in zebrafish larvae treated with different doses of flumazenil combined with 10 mg L^{-1} ($F_{7,128} = 16.05$, $p < 0.0001$), 20 mg L^{-1} ($F_{7,125} = 44.5$, $p < 0.0001$), or 30 mg L^{-1} ($F_{4,72} = 129.9$, $p < 0.0001$) diazepam. Flumazenil completely antagonized the inhibitory effects of 10 and 20 mg L^{-1} diazepam with ED$_{50}$ values of 0.086 mg L^{-1} and 1.295 mg L^{-1}, respectively (Fig. 2A and B, right). By contrast, the effect of flumazenil on 30 mg L^{-1} diazepam was weak,

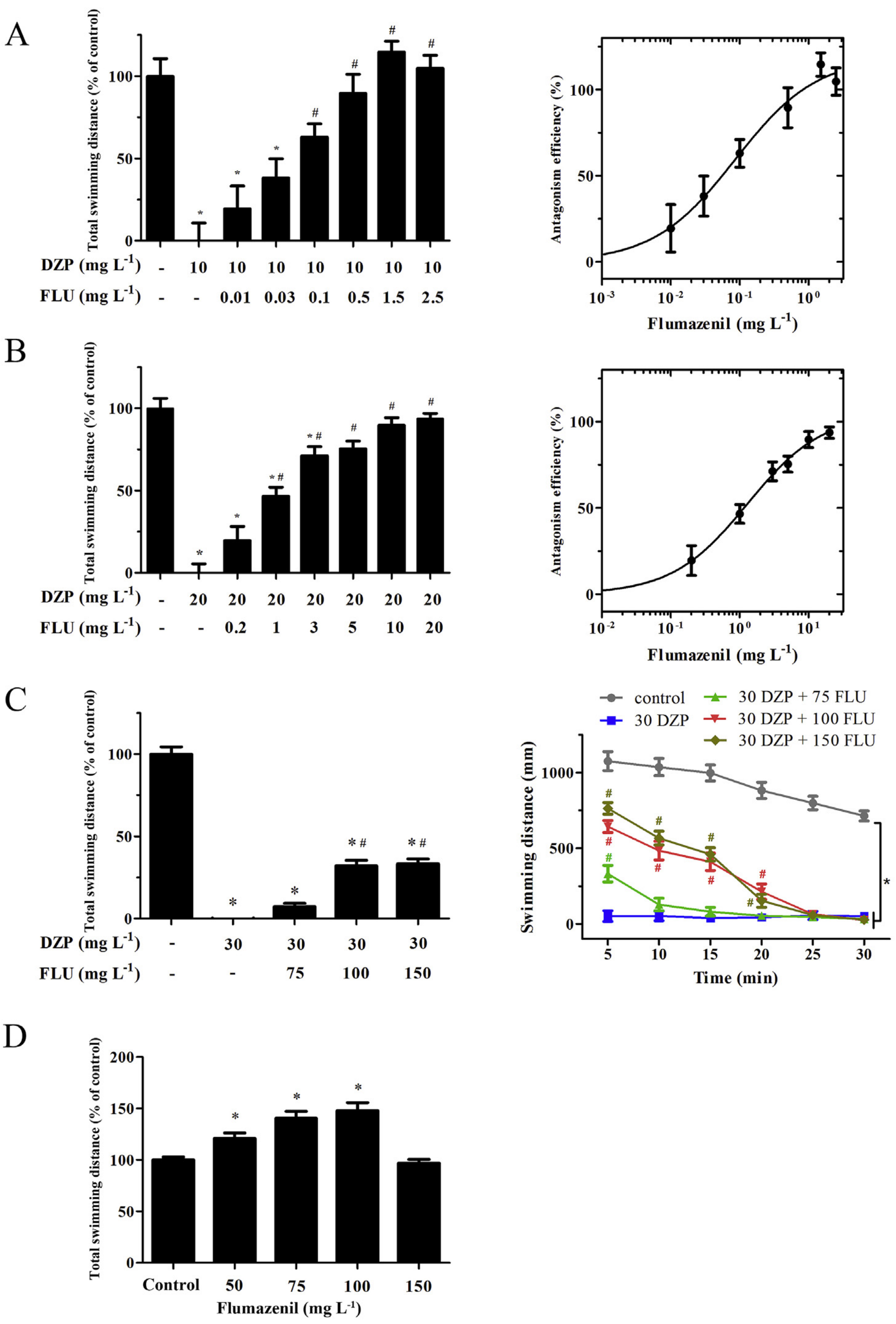

Fig. 2. Effects of flumazenil on diazepam-induced motor inhibition in zebrafish larvae. (A–B) Left, percentages of total swimming distance of zebrafish larvae treated with 10 or 20 mg L^{-1} diazepam alone or combined with different doses of flumazenil relative to that of the control group; right, dose-response curve of flumazenil antagonism of diazepam-induced motor inhibition. (C) Left, percentages of total swimming distance of zebrafish larvae treated with 30 mg L^{-1} diazepam alone or combined with flumazenil at doses of 75, 100, or 150 mg L^{-1}; right, the corresponding detailed time-course changes in the swimming distance in each 5-min bin. (D) Percentages of total swimming distance of zebrafish larvae treated with flumazenil at doses of 50, 75, 100, or 150 mg L^{-1}. Data are presented as mean (± SEM) from 40 larvae in the control group or 8–24 in the diazepam alone or diazepam combined with flumazenil-treated groups. *indicates significantly different from the control group ($p < 0.05$); $^{\#}$ indicates significantly different from the corresponding diazepam-treated group ($p < 0.05$). DZP, diazepam; FLU, flumazenil.

reverting to 7.3, 32.0, and 33.3% of the activity of the control group at doses of 75, 100, and 150 mg L^{-1}, respectively (Fig. 2C, left). The time-course change of the swimming distance of zebrafish larvae treated with 30 mg L^{-1} diazepam alone or combined with flumazenil was further analyzed. A two-way repeated measures ANOVA demonstrated significant effects of treatment ($F_{4,360} = 129.9$, $p < 0.0001$), time ($F_{5,360} = 144.4$, $p < 0.0001$), and a treatment × time interaction ($F_{20,360} = 19.12$, $p < 0.0001$) (Fig. 2C, right). Although 100 and 150 mg L^{-1} flumazenil produced statistically significant antagonism of 30 mg L^{-1} diazepam at an early stage, the antagonism was weak and declined rapidly. Moreover, flumazenil at 150 mg L^{-1} presented no augmentation of antagonism relative to 100 mg L^{-1} (Fig. 2C). The percentage of zebrafish larvae presenting an immobile state for 30 mg L^{-1} diazepam combined with 75 mg L^{-1} (56.3%), 100 mg L^{-1} (50.0%), or 150 mg L^{-1} (50.0%) flumazenil was not significantly different from that of the diazepam-treated group (62.5%) (Table 2). Flumazenil alone at these high doses produced a weak stimulatory effect on larval activity (Fig. 2D), which may be due to its weak inverse agonist properties on the GABA$_A$ receptor [24,25].

3.3. *Diazepam-induced immobility is mediated by GABA$_A$ receptors*

The flumazenil-insensitive effects of diazepam at high doses (above 30 mg L^{-1}) may be non-specific. We further investigated the antagonism of bicuculline on diazepam-induced motor inhibition. A one-way ANOVA revealed a significant difference among the locomotor activities of zebrafish larvae treated with different doses of bicuculline combined with 10 mg L^{-1} ($F_{5,47} = 14.99$, $p < 0.0001$) or 30 mg L^{-1} ($F_{5,39} = 17.29$, $p < 0.0001$) diazepam (Fig. 3A and B). In accordance with a previous report [26], bicuculline alone at 2 and 3 mg L^{-1} had no

Table 2
Behavioral characteristics of zebrafish larvae after treatment with benzodiazepines alone or combined with various antagonists.

Benzo-diazepine	Dose mg L^{-1}	Ant-agonist	Dose mg L^{-1}	Behavioral characteristics (%)			p value
				Normal	Sedation -like	Anesthesia-like	
Diazepam	10	Fluma-zenil	vehicle	57.9	42.1	0.0	–
			0.01	87.5	12.5	0.0	ns
			0.03	91.3	8.7	0.0	ns
			0.1	100	0.0	0.0	ns
	20		vehicle	45.5	45.5	9	–
			0.2	50	37.5	12.5	ns
			1	93.3	6.7	0.0	< 0.01
			3	93.3	6.7	0.0	< 0.01
			5	100	0.0	0.0	< 0.01
	30		vehicle	12.5	25.0	62.5	–
			75	0	43.7	56.3	ns
			100	0	50.0	50.0	ns
			150	0	50.0	50.0	ns
	10	Bicucu-lline	vehicle	57.9	42.1	0.0	–
			0.5	100	0.0	0.0	< 0.05
			1	100	0.0	0.0	< 0.05
	30		vehicle	0.0	14.5	85.5	–
			0.75	25.0	50.0	25.0	< 0.05
			1.5	100	0.0	0.0	< 0.01
			3	100	0.0	0.0	< 0.01
		RO 15-4513	vehicle	0.0	26.3	73.7	ns
			50	0.0	47.4	52.6	ns
			100	0.0	31.6	68.4	ns
Clonazepam	100	Fluma-zenil	vehicle	0	0	100	ns
			100	0	0	100	
Lorazepam	100		vehicle	0.0	62.5	37.5	ns
			100	0	100	0	
Trizolam	100		vehicle	66.7	33.3	0.0	ns
			100	100	0	0	
Midazolam	15		vehicle	0.0	0	100.0	< 0.01
			100	100	0	0	

Different doses of benzodiazepines alone or combined with various antagonists were added to the tank water, and zebrafish larvae behaviors were recorded during the 26–30 min after treatment. The definitions of normal, sedative, and immobile behavioral phenotypes are described in detail in the METHODS section. The differences in larvae numbers in three states between benzodiazepines alone or combined with various antagonists were compared using the Chi-square test. n = 8–24. The level of statistical significance was defined as a p value less than 0.05. ns, not significantly different.

significant influence on larval activity during the test period. However, bicuculline completely antagonized diazepam-induced motor inhibition at 10 and 30 mg L^{-1}. Furthermore, the behavior of zebrafish larvae treated with diazepam combined with bicuculline gradually reverted to normal levels as the dose of bicuculline increased (Table 2). These results suggest the existence of flumazenil-insensitive and low-affinity diazepam binding sites in $GABA_A$Rs that may mediate the strong inhibitory effects of diazepam at higher doses.

3.4. *Diazepam-induced immobility may be not mediated by sites located at the $\alpha_{4/6}/\beta_3$ interface of extrasynaptic $GABA_A$Rs*

Previous studies on the recombinant $\alpha_{4/6}\beta_3\delta$ $GABA_A$R subtype reported the existence of another low-affinity BZD-binding site at the $\alpha_{4/6}+/\beta_3$-interface of extrasynaptic receptors, which is sensitive to the imidazobenzodiazepine Ro 15–4513 [19,27]. Thus, we assessed the influence of Ro 15–4513 on diazepam-induced motor inhibition at 30 mg L^{-1} (Fig. 3C). A one-way ANOVA indicated that the locomotor activities of zebrafish larvae treated with 30 mg L^{-1} diazepam alone or combined with Ro 15–4513 (50 or 100 mg L^{-1}) was significantly different ($F_{5,103} = 189.0, p < 0.0001$). Ro 15–4513 (at doses that did not inhibit larval activity) partially antagonized diazepam-induced hypolocomotion at 30 mg L^{-1}, reverting larval activity to 35.3% and 12.6% of the control group at doses of 50 and 100 mg L^{-1}, respectively (Fig. 3C). In addition, the percentages of larvae maintained in an immobile state for 30 mg L^{-1} diazepam combined with 50 or 100 mg L^{-1} Ro 15–4513 were 52.6% and 68.4%, respectively, which was not significantly different from that of the diazepam-treated group (73.7%) (Table 2). These results suggest that Ro 15–4513 only produces weak antagonism of the high-dose action of diazepam.

3.5. *The chemical structure of BZDs influences their interaction with flumazenil-insensitive binding sites*

The effects of flumazenil on motor inhibition induced by other chemically-distinct BZDs, including clonazepam, lorazepam (both belong to 1,4-BZDs), triazolam (triazolobenzodiazepines), midazolam (imidazobenzodiazepine), and zolpidem (a non-BZD that selectively activates the classical BZD-binding site of the $\alpha_1\beta_2\gamma_2$ subtype receptor) were investigated (Fig. 4). A one-way ANOVA demonstrated a significant difference among the locomotor activities in the presence of different concentrations of clonazepam ($F_{4,35} = 98.46$, $p < 0.0001$), lorazepam ($F_{4,34} = 61.68$, $p < 0.0001$), triazolam ($F4,33 = 25.81$, $p < 0.0001$), midazolam ($F_{4,48} = 98.85$, $p < 0.0001$), or zolpidem ($F_{4,35} = 7.525$, $p < 0.0001$). It was further revealed that clonazepam, lorazepam, and midazolam dose-dependently reduced larval activity (Fig. 4A, B, and D) and increased the percentage of larvae entering an immobile (anesthesia-like) state (Table 1). By contrast, both triazolam and zolpidem produced weak motor inhibition even at 100 mg L^{-1} (Fig. 4C and E). Flumazenil at 100 mg L^{-1} effectively antagonized the inhibitory effects induced by triazolam, midazolam, and zolpidem at the highest dose, partially antagonized the hypolocomotion induced by lorazepam, but failed to affect clonazepam-induced immobility (Fig. 4, Table 2).

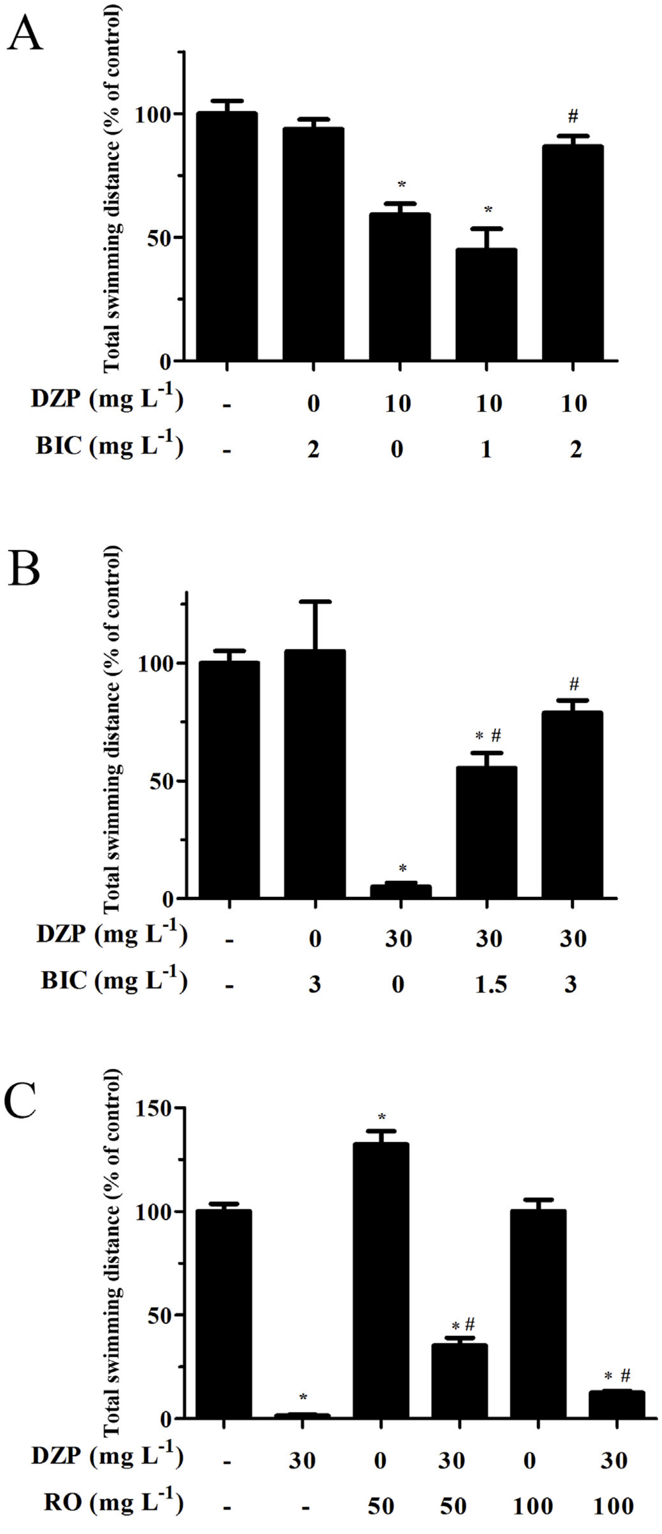

Fig. 3. Effects of bicuculline and Ro 15–4513 on diazepam-induced motor inhibition in zebrafish larvae. (A–C) Percentages of total swimming distance in zebrafish larvae treated with diazepam, bicuculline, or Ro 15–4513 alone or with combinations of these drugs relative to the control group. Data are presented as mean (± SEM) from 8 to 24 larvae in each group. *indicates significantly different from the control group ($p < 0.05$); $^{\#}$indicates significantly different from the corresponding diazepam-treated group ($p < 0.05$). DZP, diazepam; BIC, bicuculline; Ro, Ro 15–4513.

4. Discussion

Although several *in vitro* studies proposed a non-classical BZD-binding site in the $\alpha_1\beta_2\gamma_2$ subtype receptor that mediates a second potentiation of GABA-elicited currents by diazepam [9,10], nearly no *in vivo* evidence exists to support these suppositions [28]. In the present study, we found that diazepam-induced immobility at a high dose ($30\,mg\,L^{-1}$) in zebrafish larvae was resistant to flumazenil but abolished by bicuculline; another 1,4-BZD clonazepam presented similar pharmacological characteristics. These results support the existence of flumazenil-insensitive binding sites in GABA$_A$Rs that may contribute to the high-dose action of BZDs.

Through working with zebrafish, we found that diazepam produced two forms of inhibitory effects on locomotion in zebrafish larvae. Larvae exposed to diazepam at concentrations below $20\,mg\,L^{-1}$ mainly presented motor reduction, which is similar to the response in adult zebrafish and consistent with the diazepam-induced sedative-like behavioral responses in mice [29,30]. When treated with diazepam at concentrations above $30\,mg\,L^{-1}$, most larvae stopped moving at a very early stage (< 5 min) and retained immobility subsequently at the bottom of the well. More importantly, these distinct behaviors induced by diazepam showed different sensitivity to flumazenil and bicuculline. The sedative phenotype of the larvae was prevented by flumazenil, suggesting that it is mediated by classical BZD-binding sites [9,29]. By contrast, flumazenil, even at $150\,mg\,L^{-1}$, which is more than 1700-fold of the ED_{50} of flumazenil to antagonize diazepam-induced sedation, failed to effectively antagonize diazepam-induced immobility. Nonetheless, the abolishment of high-dose diazepam action by bicuculline suggests that it is mediated by GABA$_A$Rs [10]. Furthermore, the non-classical effect was not exclusive to diazepam. Other BZDs, such as clonazepam, also produced flumazenil-insensitive inhibition at higher concentrations. Collectively, non-classical, low-affinity BZD-binding sites in GABA$_A$Rs may possess a sensitivity to some 1,4-BZDs. These findings provide a possible explanation for the insufficient antagonism of flumazenil to treat certain BZD overdose in the clinic, and may call for a novel therapeutic strategy other than just blocking the classical binding sites [31–33].

To date, two possible non-classical BZD-binding sites in GABA$_A$Rs have been proposed. One is reported to be located in the $\alpha+/\beta-$ interfaces of the $\alpha_1\beta_2\gamma_2$ and $\alpha_{4/6}\beta_3\delta$ subtypes of GABA$_A$Rs [9,19,27,35]. Binding of flurazepam at a high concentration to the α_1+/β_2- site inhibits its potentiation of GABA-elicited currents at low concentrations [9,34]. Here we demonstrated that Ro 15–4513, a high-affinity antagonist of the classical $\alpha+/\gamma_2-$ site, which also exhibits antagonistic effects on the $\alpha_{4/6}+/\beta_3-$ site in $\alpha_{4/6}\beta_3\delta$ receptors [19,27,35], produced a minimal effect on diazepam-induced immobility. These results suggest that the $\alpha+/\beta-$ site may not be involved in the anesthetic effect of BZDs.

Another non-classical binding site for BZDs was shown to be located in the second transmembrane domain (TMD_2) of the $\alpha_1\beta_2\gamma_2$ subtype receptor and includes three amino acid residues (α_1S269, β_2N265, and γ_2S280), as simultaneous mutation of these residues abolishes the second concentration-dependent potentiation of diazepam on GABA-elicited currents [9]. Notably, these residues have also been demonstrated to be important for the allosteric modulation of GABA$_A$Rs by many clinical anesthetics, including etomidate and propofol [36]. After genetic sequence comparison, we noticed that zebrafish possess conserved amino acid alignments in the TMD_2 of the various GABA$_A$R subunits (except the α3 subunit) compared to those of rats, especially the key ternary residues (Supplementary Fig. S1). Therefore, the flumazenil-insensitive BZD effects observed in our study are likely to be mediated by the non-classical site in TMD_2. However, the gene sequence homology of GABA$_A$R subunits implies that these sites may be located in many GABA$_A$R subtypes, making it technically challenging to knockout or mutate all of them simultaneously. Recently, several novel compounds targeting these TMD_2 binding sites were designed.

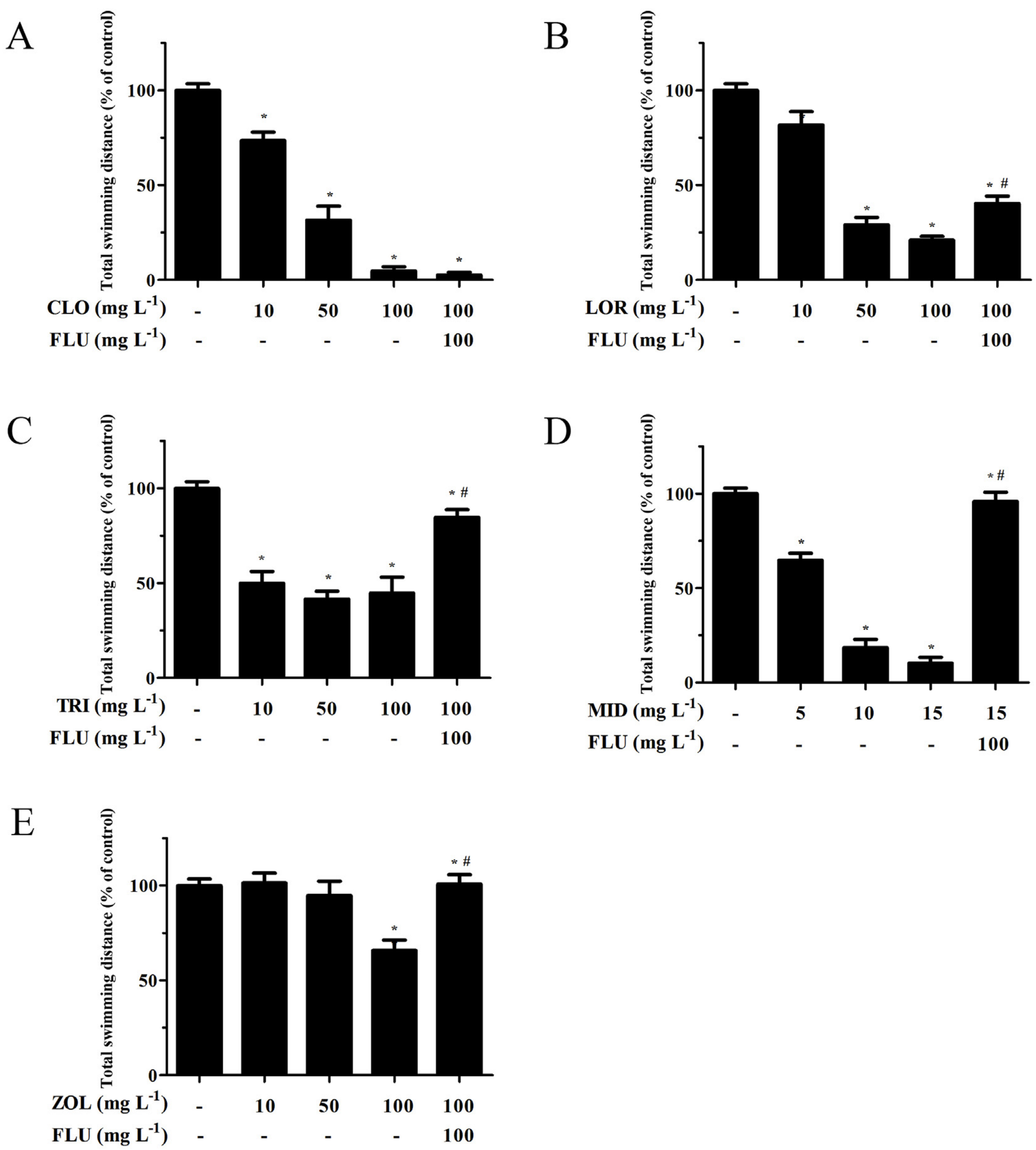

Fig. 4. Effects of flumazenil on BZD-induced motor inhibition in zebrafish larvae. (A–E) Percentages of total swimming distance of zebrafish larvae treated with clonazepam, lorazepam, midazolam, triazolam, and zolpidem alone or combined with 100 mg L^{-1} flumazenil. Data are presented as mean (± SEM) from 8 to 24 larvae in each group. *indicates significantly different from the control group ($p < 0.05$); $^{\#}$indicates significantly different from the corresponding BZD-treated group ($p < 0.05$). CLO, clonazepam; LOR, lorazepam; MID, midazolam; TRI, triazolam; ZOL, zolpidem; FLU, flumazenil.

Compound 31 and 132 produced powerful potentiation of GABA-elicited currents [37], while flavan Fa173 blocked diazepam-induced electrophysiological responses at a high concentration [33]. These compounds, after further validation, will be useful to draw a precise conclusion on whether the proposed low-affinity BZD-binding sites are located in the TMD_2 of $GABA_A$Rs.

5. Conclusion

Our study supports the concept of at least two distinct binding sites for BZDs located in $GABA_A$Rs [9,10]. Besides the classical high-affinity sites mediating low-dose diazepam actions, such as sedation, there is another non-classical low-affinity site possibly located in the TMD_2 of the receptor that contributes to the anesthetic effects of BZDs. Further, we provide a convenient and high-throughput behavioral model to investigate non-classical BZD-binding sites in $GABA_A$Rs.

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Contributors

Yanqing Cao, Gang Yu and Ruibin Su design the study and write the manuscript; Yanqing Cao and Hui Yan conduct the experiments; Yanqing Cao and Gang Yu analysis the data.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

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