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$GABA_A$ receptors activate fish feeding behaviour via two distinct functional pathways

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Summary statement

This study combines behavioural tests with cellular and molecular electrophysiology to clarify molecular mechanisms that control fish feeding. Two partially competitive mechanisms originating from the same neural receptor were found.

Abstract

Benzodiazepines, acting through ionotropic receptor of γ-aminobutyric acid ($GABA_A$ receptor, GABAR), were shown to modify feeding behaviour and increase appetite in humans and non-human subjects. However, the cellular and molecular mechanisms which underlie connected short-term behavioural fluctuations are still unclear. In the present study, we used *Carassius gibelio* (Prussian carp) as a model organism to research the impact of scantily explored benzodiazepine phenazepam (PNZ) on feeding behaviour and the related molecular mechanisms of PNZ action at single-cell and single-receptor levels. We found that the feeding activity of *C. gibelio* is under control of GABARs via two distinct mechanisms: orthosteric (triggered by GABA binding site) and allosteric (triggered by benzodiazepine binding site). PNZ displayed clear stimulatory effects on both mechanisms in GABA-dependent manner. On top of this, orthosteric and allosteric effects were found to be partially competitive, which leads to complex behavioural repercussions of conjoint effects of GABAR ligands.

Key words: $GABA_A$ receptor, feeding, *Carassius gibelio*, benzodiazepines

Introduction

Over recent decades there has been tremendous progress in deciphering how the nervous system modulates feeding behaviour and appetite. Cellular and molecular studies demonstrated that γ-aminobutyric acid (GABA), a major inhibitory neurotransmitter, plays a critical role in feeding behaviour regulation (Sohn et al., 2013; Williams and Elmquist, 2012). When activated, ionotropic $GABA_A$ receptors (GABARs) commonly induce appetitive increases and upregulate feeding activity (Reynolds and Berridge, 2001; Wu et al., 2009).

Benzodiazepines, which act as allosteric GABAR agonists, have long been accepted as a class of compounds that induces hyperphagia and activates appetite after binding to GABARs. This effect has been established in a number of animal species (Baile and McLaughlin, 1979; Brown et al., 1976; Cooper et al., 1985; Gaskins et al., 2008) and in humans (Haney et al., 1997), where benzodiazepines were shown to control abnormal feeding activity and obesity (Fieldstone et al., 1998).

In contrast to Tetrapoda, benzodiazepines effect in lower animal taxa is much less studied. In particular, to the best of our knowledge, there is no data on changes in fish feeding behaviour induced by benzodiazepines, despite this class of compounds having been reported to upregulate overall food consumption when continuously present in water (Belal and Assem, 2011; Brodin et al., 2013). On top of that, to the best of our knowledge, there is no or little information on the behavioural repercussions of interaction between benzodiazepines and other GABAR ligands in fishes.

A number of marine and fresh-water fish species were introduced as experimental models in food consumption research, due to their relatively simple and easily quantifiable feeding behaviour (Facciolo et al., 2010; Matsuda, 2009; Yamashita et al., 2006). In particular, *Carassius gibelio* (Prussian carp) has been established as a popular animal model for research of GABAR-mediated effects on behaviour (Facciolo et al., 2011; Volkoff, 2013), which provides a rationale for our choice of this species as an experimental object.

A contemporary approach to *in vivo* receptor modulation and drug design implies combined usage of compounds with different types of molecular action mechanisms: orthosteric (competing for binding site with endogenous ligand) and allosteric (not competing with endogenous ligand). Combinations of different ligand types typically afford unprecedented levels of selectivity and fine tuning of end effects (Christopoulos, 2002; Wenthur et al., 2014). Thus, due to allosteric action mechanism of benzodiazepines, an important research direction regarding their pharmacological properties is investigation of mutual modulation between benzodiazepine effect and effects caused by GABAR orthosteric ligands.

Taken together, these facts imply the need for behavioural study combined with research of benzodiazepine effects at cellular and molecular level to clarify benzodiazepine-controlled molecular mechanisms of GABARs modulation and correlate them with a profile of behavioural response. Since this approach aggregates data generated at different levels of organisation (cellular, molecular and whole organism), it provides an opportunity for a complex characterisation of perspective chemical compound(s) that have not yet been fully explored.

Phenazepam [7-bromo-5-(2-chlorophenyl)-1,3-dihydro-2H-1,4-benzodiazepine-2-one] (PNZ), is a benzodiazepine which was synthesized in the USSR in 1974 (Bogatskii et al., 1975) and was never in clinical use outside of former Soviet territory. There is scarce data available on PNZ pharmacology, pharmacokinetics, and molecular mechanisms of action, and even less about its impact on behaviour (Maskell et al., 2012); however, it was proven to exert a significant modulatory effect on GABA-ergic inhibitory currents (Kopanitsa et al., 2002) and weaken chemical stress reactions in fishes (Potrohov, 2008). Therefore, we

selected PNZ for our study as an under-examined allosteric agonist allowing modulation of GABAR-conducted currents and promising a significant impact on the behavioural profile of *C. gibelio*. We set out to test whether and to what extent fish feeding behaviour is controlled by PNZ action, to relate this data to cellular and molecular effects of benzodiazepine binding site activation, and to clarify, in this context, the functional interaction of modulatory effects induced at orthosteric (GABA-binding) and allosteric (benzodiazepine-binding) sites of GABAR.

Methods and materials

Animals. All experiments were performed on wild-type *C. gibelio* (Prussian carp), body length 10-15 cm, of either sex. Fish were maintained, and all experiments were performed at room temperature (22-25 °C). Before treatment fish were allowed to acclimate for two weeks under a 12L:12D photoperiod in flow-through constantly aerated tanks (60 l). Fish were fed once a day with commercial granulated food. Animal maintenance and experimental procedures were conducted in accordance with the Guide for Use and Care of Laboratory Animals (European Communities Council Directive, 1986) and UK Animals (Scientific Procedures) Act 1986.

Behavioural tests. After acclimation period fish were randomly placed into testing tanks shielded by white paper sheets to minimize external disturbance. Subsequently, the animals were lightly anesthetized in ice water (Facciolo et al., 2011) and injected intraperitoneally with GABAR ligands. Injections were administered caudal to the pelvic fin. To verify that the injections themselves did not influence feeding behaviour, we performed a control test where un-handled fish (n=6) and fish injected with 0.1 ml of 0.9% NaCl (n=6) were compared by feeding behaviour during 8 hours. On the basis of similar behavioural responses (average number of feeding acts) between groups, saline-treated animals were considered as controls for all behavioural experiments. One more control test was performed to determine when fish can be returned into experimental use. In this test we compared feeding behaviour of control fish group and fish groups subjected to injections of 0.3 mg/kg PNZ, 3 mg/kg Bic and 3 mg/kg SR. No difference in feeding acts number was found after three days post injection. We therefore used each fish for injection of single dose of GABAR ligands in 4-6 days after the end of previous experiment.

Feeding behaviour was monitored and quantified as described previously (Volkoff, 2013). Briefly, a “feeding act” (FA) was defined as an event when the fish consumed food granule (FAC), or engulfed a granule and then “spat” it out (FAS), or pushed a granule with closed mouth (FAP).

For the evaluation of the GABAR effects on the feeding behaviour (number of FAs) fish were injected with GABAR ligands diluted in 0.9% NaCl vehicle and compared to animals that received an i.p. injection of vehicle only (controls). Episodes of convulsive behaviour after injection of GABAR antagonists were defined as those where intermittent rapid swimming in circles and/or sporadic rapid swimming with abrupt changes of direction were observed (Facciolo et al., 2011). Immobility episodes after injection of benzodiazepine were defined as those where stillness was observed for ≥10 min (Ide and Hoffmann, 2002).

Electrophysiology. To prepare brain slices fish were anesthetized with 130 mg/l MS-222 (Posner et al., 2013), and then rapidly decapitated. The brain was rapidly removed, placed into agar block and transferred to Leica VT1000 vibratome slicing chamber. For slice preparation, we used telencephalon as a part of fish brain which is tightly connected to feeding behaviour (Lin et al., 2000), and where GABAR response in *C. gibelio* was clearly demonstrated (Hossein-Javaheri et al., 2017).

Electrophysiological patch-clamp recordings were performed with MultiClamp 700B amplifier (Molecular Devices) under Clampex 10x software, using 4-6 MΩ borosilicate glass electrodes at room temperature (22-25 °C). Perfusion media contained (in mM): NaCl 108, KCl 2.5, $MgCl_2$ 2, CaCl 2.5, $NaHCO_3$ 24, NaH_2PO_4 1, glucose 12, gassed with 95%O_2-5%CO_2, pH adjusted to 7.4 with NaOH. Slicing media differed from perfusion media by concentrations of $CaCl_2$ (0.5 mM) and glucose (5 mM). Intracellular electrode solution contained (in mM): CsCl 115, HEPES 25, Mg-ATP 3, Na-GTP 0.5, EGTA 0.5, Tetraethylammonium chloride 10, pH 7.2. To isolate GABAR response we added to perfusion solution (in μM): APV 50, NBQX 20, MCPG 250, CGP-55845 1, strychnine 1. GABAR ligands (SR-95531, bicuculline, phenazepam, flumazenil, picrotoxin) were added to perfusion solution in a course of experiment. SR-95531, bicuculline, picrotoxin, strychnine, MCPG, NBQX, CGP-55845, APV and flumazenil were purchased from Tocris Bioscience; phenazepam was synthetized and kindly provided by Bogatskii Physico-Chemical Institute (Odesa, Ukraine); all other chemicals were purchased from Sigma-Aldrich.

Decay profiles of IPSCs recorded in whole-cell mode were fitted with double-exponential function

$$\Delta I = -a_1 e^{-\frac{t}{\tau_1}} - a_2 e^{-\frac{t}{\tau_2}}$$

where ΔI is a difference between current recorded at baseline and at time *t*, e – Euler's constant, a_1 and a_2 – fitting constants, τ_1 and τ_2 – decay time constants.

Charge transfer per second in single-channel recordings from outside-out patches was calculated as $I/U \times \Delta t$, where I is an amplitude of single-channel response (pA), U – holding membrane potential (mV), Δt – time spent in an open state (ms). Time intervals when GABARs are open were selected using a detection threshold of 1.5 pA more negative than mean baseline and a minimum opening time of 0.2 ms. Values for the open time of single channels were obtained with threshold-detection algorithm of Clampfit 10x software.

Nonlinear fitting and statistical calculations were performed with Mathematica-10 and -11 software packages. Data are presented as mean±SEM and evaluated for statistical significance with Student's paired or unpaired t-test, as indicated. Dose-response effects on behaviour elements were assessed with one-way ANOVA; in time-response behavioural experiments two-way ANOVA followed by Bonferroni post hoc test was used for comparison between groups.

Results

Behavioural tests

To determine most effective doses for GABAR ligands, we performed a "dose-response" study (Fig. 1). In dose-response experiments with 2 hours observation interval starting 15 minutes after drug injection, we aimed to find a GABAR ligand dose that produces the strongest effect without immobility episodes (PNZ) or episodes of convulsive behaviour (GABAR antagonists). Effect of GABAR ligands was tested at overall number of FAs and separately for FAC, FAS and FAP number: see Table 1 for one-way ANOVA results.

PNZ demonstrated a significant gradual increase of number of FAs with incrementing dosage (Fig. 1A), but without significant effect on any of separate FA components (FAC, FAS, FAP). Dose 0.2 mg/kg was the highest when fish did not demonstrate immobility episodes; on top of that, higher doses did not generate number of FA significantly higher than that for 0.2 mg/kg. Competitive GABA antagonist SR-95531 (SR) and inverse agonist with partial allosteric effect bicuculline (Bic) displayed decrease of FA number with incrementing dosage; the highest effect without convulsion episodes was produced by 2 mg/kg SR and 1.5 mg/kg Bic (Fig. 1B and C). In both cases FAC and FAS, taken alone, were found to be significantly influenced by incrementing doses of GABAR ligands (Table 1). Flumazenil (FMZ; allosteric antagonist acting at benzodiazepine binding site), being injected in up to 5 mg/kg dose, did not generate a significant effect on overall FA number as well on FAC, FAS and FAP components taken separately (Fig. 1D, Table 1). Therefore, for further *in vivo* experiments, we used doses of 1.5 mg/kg for Bic, 2 mg/kg for SR and 0.2 mg/kg for PNZ. FMZ, being injected alone, did not display significant effects in any dose; thus taking

into account data from the literature (up to 40 mg/kg dosage in *in vivo* experiments (Petry and Heyman, 1997)), we have chosen a highest tested dose of FMZ (5 mg/kg) for further behavioural experiments. Since the overall number of FAs was the only parameter which demonstrated significant dependence from three GABAR ligands (Table 1), we used it as an assessment criterion in further tests.

To find the optimal time course for combined injections of GABAR ligands, we performed a "time-response" behavioural experiment with optimal ligand doses determined earlier (Fig. 2A). We found that PNZ develops a maximum effect 1.5 hour after injection, whereas SR and Bic do so in 1 hour; again, FMZ did not display significant difference from control during all the period of observation.

Next, we tested combined effects of GABAR ligands on *C. gibelio* feeding behaviour. To do this, we first injected PNZ and, 0.5 hour later (to overlap peak responses), 1.5 mg/kg of Bic, plus different doses of SR (Fig. 2B). Unexpectedly, a low dose of SR reversed Bic antagonism to PNZ: $F(1,90)=4.22$, $P=0.042$, two-way ANOVA for 0.3 mg/kg of SR; whereas a higher dose (0.5 mg/kg) potentiated the Bic effect. To find out whether this paradoxical observation was due to modulation caused by allosteric site after binding of PNZ, we repeated the experiment without PNZ pre-injection (Fig. 2C). In this case, incrementing doses of SR monotonically enhanced the Bic effect in a dose-dependent manner. 0.3 mg/kg of SR did not cause a significantly different effect from that evoked by Bic only: $F(1,96)=3.71$, $P=0.057$, two-way ANOVA. However, 0.3 mg/kg of SR evoked convulsive episodes in 4 out of 6 experimental animals; we thus did not continue the experiment with higher SR doses.

Dependence of Bic+SR effects from presence of PNZ confirmed involvement of benzodiazepine binding site into reversal of Bic effect caused by SR. To further test this, we performed an experiment where 1.5 mg/kg of Bic and incrementing doses of SR were injected 0.5 h after combined injection of PNZ and FMZ (Fig 2D). Here Bic plus 0.3 mg/kg SR generated significantly higher response than Bic alone ($F(1,90)=6.86$, $P=0.01$, two-way ANOVA); convulsive episodes were observed in 3 out of 6 animals. Again, in this experiment SR enhanced Bic effect in a dose-dependent manner, thus resembling data from the experiment where SR and Bic were used without PNZ.

Equipped with this knowledge, we formulated a working hypothesis explaining the effects of SR, Bic and PNZ as follows. SR and Bic, applied together, compete for the same binding site with endogenous GABA; their joint effect is thus proportional to the number of occupied GABA-binding sites at GABARs and, therefore, increases continuously when SR concentration increments. When applied after PNZ, Bic evokes both a competitive effect

(displacing GABA at conjoint binding site) and an allosteric effect (counteracting conformational changes induced by PNZ). In contrast, SR in small doses, after dislodging of Bic from the binding site, delivers only competitive impact without counteraction to allosteric effect of PNZ, thus weakening joint antagonist effect on GABARs; however, incrementally increasing doses of SR occupy additional GABA-binding sites and, thus, start enhancing the effect of Bic.

Therefore, we proceeded to electrophysiological experiments aiming to test the working hypothesis. To do this, we aimed to dissect effects of PNZ, Bic and SR and their mutual modulation at whole-cell and single-receptor level.

Electrophysiology

As a first part of the electrophysiological section of our study, we performed recordings of spontaneous synaptic GABA-ergic responses in whole-cell mode. We presumed that the decay profile of spontaneous inhibitory post-synaptic currents (s-IPSCs) should consist of two exponents, with one exponent generated by orthosteric (GABA-binding) site effects, and another by effects of the allosteric (benzodiazepine-binding) site. Thus, for quantification of effects generated at orthosteric and allosteric sites, we used the ratio of fitting coefficients (RFC) of two IPSC decay exponents obtained after fitting of averaged s-IPSCs with bi-exponential function (see Fig. 3D legend for more details of analysis paradigm). Picrotoxin (PTX, 50 μM), applied at the end of each experiment, fully suppressed all synaptic events, thus confirming their exclusive GABA-ergic origin.

Application of PNZ (0.2 μM) increased s-IPSC amplitudes and significantly slowed down their decay kinetics (Fig. 3A and C); RFC 3.62±0.64 for PNZ vs. 6.03±0.68 in control, P=0.016, n=6, paired Student's t-test. SR (50 nM), added together with PNZ, did not influence response decay kinetics biased by PNZ: RFC 2.87±0.47 for PNZ+SR vs. 3.62±0.64 for PNZ alone, P=0.235, n=6, paired Student's t-test. In contrast, Bic (50 nM), when applied with PNZ, restored s-IPSC decay kinetics to almost control values: 5.56±0.42 for PNZ+Bic vs. 3.62±0.64 for PNZ alone, P=0.029, n=6, paired Student's t-test (Fig. 3E). RFC analysis (Fig. 3D) demonstrated that PNZ application magnified slow decay exponent, which then remained unaltered by SR, but suppressed by Bic.

This confirmed the different nature of the antagonism to PNZ effect generated by SR and Bic. However, was it due to modulation of the benzodiazepine binding site by one of these antagonists? To clarify the issue, we repeated the experiment on s-IPSCs, but with FMZ applied before Bic (Fig. 4). FMZ (2 μM) added after PNZ, reverted the bias of RFC caused by PNZ: 5.73±0.69 for PNZ+FMZ vs. 3.51±0.71 for PNZ alone, P=0.0009, n=6, Student's

paired t-test. Bic, added to perfusion solution after FMZ, did not cause a significant effect: RFC 5.05±0.77 for PNZ+FMZ+Bic vs. 5.73±0.69 for PNZ+FMZ, P=0.55, n=6, Student's paired t-test (Fig. 4C).

We thus concluded that the effects of Bic on decay kinetics were due to its impact on benzodiazepine binding site when the site is activated by PNZ; FMZ, displacing PNZ, prevents the effect of Bic.

Next, to test whether Bic generates an allosteric effect when the benzodiazepine site is inactive, we performed an experiment on s-IPSCs without benzodiazepine site ligands (Fig. 5). In this case, both SR and Bic did not show a significant impact on s-IPSC kinetics: RFC 5.29±0.83 for control, 6.21±0.67 for SR, 5.9±0.78 for Bic; P>0.05 for all comparisons, n=6, Student's paired t-test.

Experiments on s-IPSC kinetics confirmed our working hypothesis about the interaction of GABAR antagonists (SR and Bic) with allosteric benzodiazepine binding site. However, in all these tests, the orthosteric site of the receptor was (possibly) activated by endogenous GABA. Thus, to further dissect mechanisms of action of benzodiazepines and GABAR antagonists, and to clarify their input through the GABAR orthosteric site, we proceeded to single-channel recordings from outside-out patches, where GABA presence and/or concentration can be tightly controlled. Here we used an overall charge transfer per second as a quantitative characteristic of output generated by pharmacological manipulations.

We found that GABA (1 μM), when added to perfusion solution, induced a clear response which was suppressed by both SR (20 μM) and Bic (20 μM) to values similar to that obtained for spontaneous openings (Fig. 6A and C). Charge transfer normalized to that generated by GABA only was 0.11±0.03 for control (spontaneous openings), 0.16±0.03 for GABA+SR, 0.09±0.01 for GABA+Bic; P>0.05 for all comparisons, n=6, paired Student's t-test. However, when added to perfusion solution after application of PNZ (without GABA), Bic, but not SR, exerted significant downregulation on charge transfer (Fig. 6B and D): normalised charge transfer for PNZ+SR 0.98±0.14 vs. 0.6±0.09 for PNZ+Bic, P=0.041, n=6, paired Student's t-test.

Finally, we tried to reproduce the pharmacological steps from *in vivo* experiments with PNZ and FMZ on outside-out patches (Fig. 2B and D). We, thus, first applied GABA (1 μM) + PNZ (0.2 μM) and antagonized their combined effect by Bic (2 μM) and Bic (2 μM) + SR (2 μM) (Fig. 7A and C). In accord with the *in vivo* behavioural experiment, SR partially reversed the effect of Bic on charge transfer: normalized charge transfer 0.28±0.08 for GABA+PNZ+Bic vs. 0.52±0.07 for GABA+PNZ+Bic+SR, P=0.002, n=6, Student's paired t-test. Second, we

applied GABA (1 μM) + PNZ (0.2 μM) + FMZ (2 μM) and antagonized them with Bic (2 μM) and Bic + SR (2 μM) (Fig. 7B and D). In contrast to the experiment without FMZ, here, application of Bic+SR did not cause a significantly different effect from Bic alone: normalized charge transfer 0.35±0.07 for GABA+PNZ+FMZ+Bic vs. 0.31±0.09 for GABA+PNZ+FMZ+Bic+SR, P=0.68, n=6, Student's paired t-test.

Discussion

In the present work we performed a combined behavioural and *in vitro* electrophysiological study of GABAR effects generated by two distinct mechanisms: orthosteric and allosteric. Correlation of results obtained in these two types of experiments clearly demonstrates control of GABARs over *C. gibelio* feeding behaviour delivered through both mechanisms in (partially) competitive mode.

Benzodiazepines, which PNZ belongs to, after binding to GABAR initiate two types of potentiation: orthosteric (GABA-dependent) due to enhancement of GABAR affinity to GABA (Lavoie and Twyman, 1996) and allosteric (GABA-independent) through increase of GABAR opening probability (Birnir et al., 2000). A key point of our research, which all further conclusions depend on, is the nature of SR and Bic action against PNZ ortho- and allosteric effects. Bic and SR were shown to compete for the same binding site with GABA (Johnston, 2013; Wagner and Czajkowski, 2001). With different experimental approaches, both orthosteric and allosteric action mechanisms were demonstrated for these two compounds (Bai et al., 2001; Heaulme et al., 1986; Krishek et al., 1996; Thompson et al., 1999; Ueno et al., 1997). The inverse agonist impact of Bic was found to suppress GABA-independent GABAR openings (McCartney et al., 2007; Wlodarczyk et al., 2013) and antagonize effects of GABAR allosteric agonists (Mathers et al., 2007; Ueno et al., 1997). In our experiments, Bic, but not SR, exerted significant antagonism to allosteric PNZ effect when drugs were applied on outside-out patches with no GABA in perfusion solution (Fig. 6B and D). In contrast, they both antagonized the effect of GABA (Fig. 6A and C), which implies competitive action at orthosteric GABAR binding sites. We thus concluded that, in our research model, SR displays competitive (orthosteric) action only, whereas Bic generates both orthosteric and allosteric effects.

Three different types of feeding behaviour registered in our study undergo different modifications as a result of GABAR activation/deactivation (Fig. 1). Sum of all three types of feeding events (FA) was found to be most correlated with GABAR ligands action, whereas individual modes of behaviour (FS, FC, FP) may not be influenced significantly by application of particular ligand. This probably reflects the fact that behaviour types under

study are closely interrelated, and, as a consequence, easily undergo mutual transition (Helfman, 1986) within general behavioural shift due to GABAR-triggered impact.

An experiment where SR, applied alone, induced a clear decrease in the number of FA competing with endogenous GABA (Fig. 1B), confirmed the input of orthosteric functional pathway into feeding behaviour. When applied together, SR weakened the antagonistic impact of Bic on the effect of PNZ in both behavioural tests (Fig. 2B) and single-channel recordings (Fig. 7A and C), whereas FMZ, acting at allosteric site, precluded such an effect of SR (Fig. 2D and 7D). The most straightforward explanation is that Bic antagonizes both orthosteric and allosteric effects of PNZ, whereas SR, after displacement of Bic, blocks orthosteric effect of PNZ, but leaves its allosteric effect untouched. Hence, these two lines of evidence confirm our working hypothesis about the molecular mechanisms of interaction between SR, Bic, and PNZ. Paradoxical antagonism of SR to Bic effect, displayed *in vivo* and *in vitro*, unequivocally points to allosteric GABAR potentiation as one more functional pathway for modulation of fish feeding behaviour.

In experiments on s-IPSCs, PNZ application slowed down overall decay kinetics due to magnification of exponent with higher τ value (slow decay component, Fig. 3), which is in line with effects of benzodiazepines observed earlier (Poncer et al., 1996). Bic, being added after PNZ, shifted RFC due to suppression of the slow decay component. In turn, FMZ, which competes with PNZ for allosteric binding site, also suppressed the slow decay component (Fig. 4), suggesting this component to be generated as a result of allosteric site activation. Since SR was found to deliver its effect exclusively via orthosteric site of action, one may expect SR, when applied after PNZ, to generate a bias of RFC opposite to that generated by Bic: suppression of fast decay component with the slow component left intact. However, we did not observe any significant RFC shift generated by SR (Fig. 3 and 5). The plausible explanation for this phenomenon is that allosteric activation of spontaneous openings induced by PNZ (see Fig. 6B) is a stable background input, which does not affect IPSC kinetics. In contrast, increased affinity to GABA after PNZ binding, affects responses generated by GABA efflux only. Thus if both fast and slow (latter - magnified due to increased affinity to GABA) decay components are generated at orthosteric site, SR, displacing GABA, should exert proportional impact on both decay components without changes in RFC, which corresponds to our observations.

Depending on cell type and particular compound, benzodiazepines may affect phasic, tonic, or both types of GABAR-induced inhibition (Bai et al., 2001; Glykys and Mody, 2006; Nusser and Mody, 2002; Yamada et al., 2007). Tonic GABAR conductance can be produced by two functional types of receptors: GABA-dependent, i.e. activated by low concentrations

of ambient GABA (Farrant and Nusser, 2005), and GABA-independent, i.e. spontaneously opening (McCartney et al., 2007; Wlodarczyk et al., 2013). Thus, only the ability to modulate tonic conductance through spontaneously opening GABARs may prove the GABA-independent, irreducible to increasing affinity of GABAR to GABA, action of given benzodiazepine. In our experiments, we found that PNZ potentiates phasic and tonic responses of GABARs; tonic conductance was potentiated without the presence of GABA (Fig. 6), which resonates with earlier observations on classical benzodiazepine compounds such as diazepam (Birnir et al., 2000). Hence, our data prove that PNZ delivers its impact through two types of GABAR potentiation: GABA-dependent and GABA–independent.

Another observation, which looks to some extent counterintuitive, is a difference between the behavioural effect of incrementing doses of SR when PNZ was not injected (no significant difference from the effect of Bic only, Fig. 2C) and when PNZ binding was blocked by FMZ (significant difference from Bic only, Fig 2D). The possible explanation is that in the native brain benzodiazepine binding site is neither fully activated nor suppressed, and thus generates a certain amount of spontaneous receptor openings (as registered in outside-out patches, Fig. 6 and 7). SR, being added on top of Bic, enhances Bic antagonistic effect when competes for binding site with endogenous GABA, but removes Bic antagonism to partially activated benzodiazepine site when competes with Bic. These opposite actions prevent development of statistically significant end effect of certain SR doses. However, when benzodiazepine site is blocked by FMZ, competition between SR and Bic does not generate any indirect effect connected to riddance of Bic allosteric action, thus allowing unhindered development of antagonism due to GABA displacement by SR. If this is the case, this also implies regulation of feeding behaviour by two types of GABAR signalling: phasic (synaptic IPSCs generated by GABA efflux) and tonic (spontaneous GABAR openings all over the cell surface).

Our study thus has several outcomes. As a fundamental result, we proved existence of two distinct molecular mechanisms regulating feeding behaviour through GABARs, which act in partially competitive manner. Due to relatively conservative nature of GABAR structure in vertebrates (Martyniuk et al., 2007), these two mechanisms with given interaction should be expected in higher taxons as well as in fishes. If true, this makes *C. gibelio* a convenient and universal experimental model for further studies of GABAR effects on feeding behaviour. On top of that, we filled a data gap in behavioural effects of PNZ which is perspective, albeit under-researched compound for treatment of GABAR-connected disorders.

After impetuous growth during last decades, aquaculture now supplies more than a half of the total fish for human consumption; in parallel, aquaculture's share of the world’s fishmeal

consumption more than doubled (Naylor et al., 2009). Further progress in fish aquaculture is thus tightly connected to intensification and optimisation of feeding of the cultivated fishes; this is a subject of intense studies in particular on species of *Carassius* genus (Wang et al., 2015; Xue and Cui, 2001). Therefore, the applied outcome of our study is highlighting of benzodiazepines as perspective compounds for use in aquaculture as stimulants of fish feed intake and weight gain.

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Author contributions: S. Snigirov participated in experimental work and provided critical review of the paper; S. Sylantyev participated in experimental work, performed numerical calculations and prepared the paper; both authors developed concept of the study, working hypotheses and experimental design.

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Table 1. One-way ANOVA output for dose-response relationships between GABAR ligands and different types of feeding act.

Ligand	Behavioural element	Degrees of freedom	F ratio	P value*
PNZ	All FAs	6, 50	3.36	**0.007**
	Pellet consumed		2.16	0.062
	Pellet spat out		2.09	0.071
	Pellet pushed		2.21	0.057
SR	All FAs	6, 50	2.72	**0.023**
	Pellet consumed		2.68	**0.025**
	Pellet spat out		3.95	**0.003**
	Pellet pushed		1.47	0.207
Bic	All FAs	6, 52	3.71	**0.004**
	Pellet consumed		2.92	**0.016**
	Pellet spat out		5.99	**8.2×10^{-5}**
	Pellet pushed		1.74	0.13
FMZ	All FAs	6, 47	0.24	0.961
	Pellet consumed		0.69	0.658
	Pellet spat out		1.2	0.323
	Pellet pushed		2.23	0.057

* - P<0.05 are marked by bold.

Figures

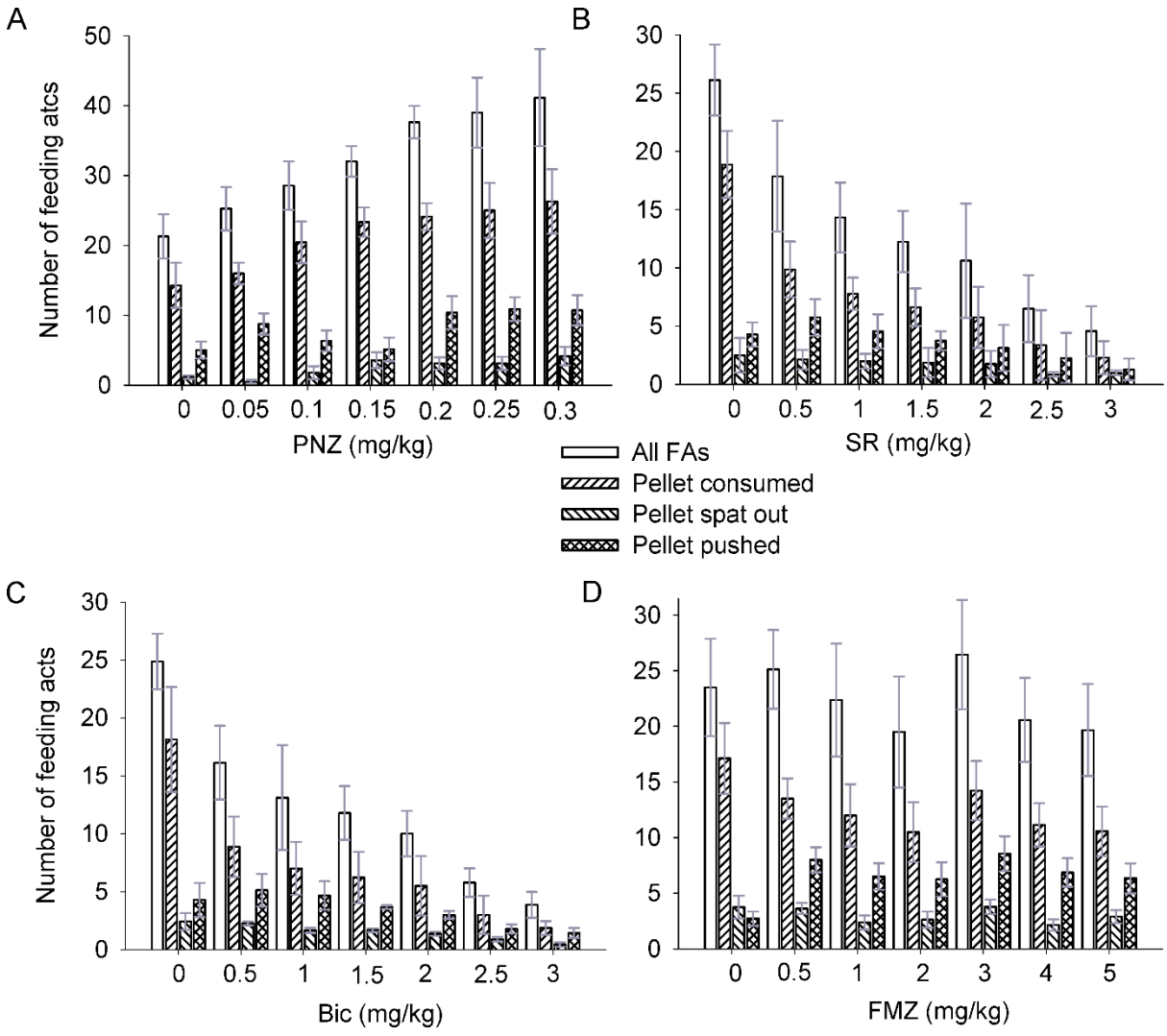

Figure 1. GABAR ligands of different action mechanisms modulate fish feeding behaviour. Dose-response relationships of feeding behaviour elements were registered 15 min after i.p. injection during 2 hours' time intervals. Bar pattern codes apply to A-D.

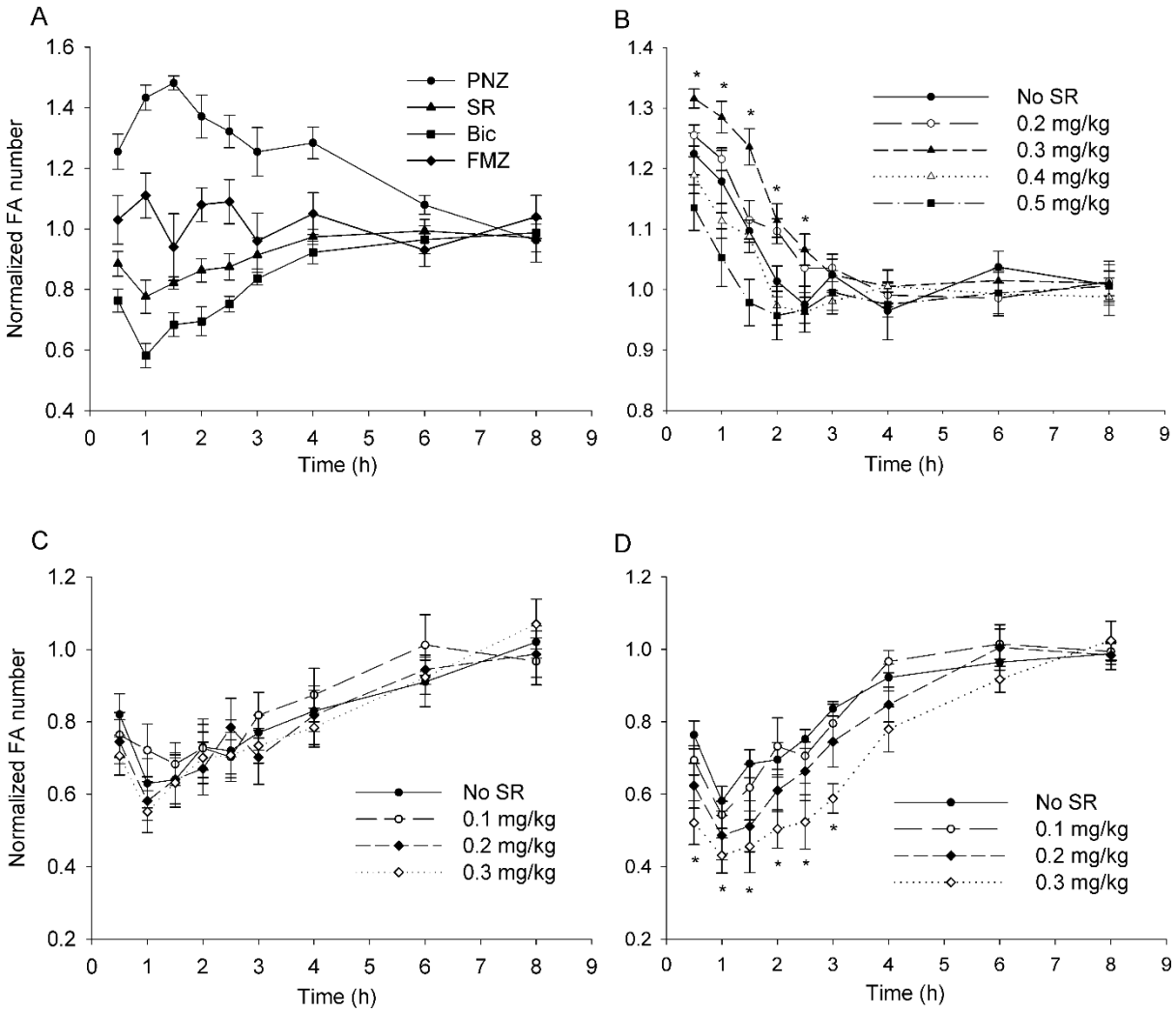

Figure 2. SR reverses Bic antagonism to PNZ effect on behaviour. A: Time-response relationship for four GABAR ligands: 0.2 mg/kg PNZ, 2 mg/kg SR, 1.5 mg/kg Bic, 5 mg/kg FMZ. **B:** Bic (1.5 mg/kg) and SR were injected 30 minutes later than PNZ (0.2 mg/kg). **C:** Bic (1.5 mg/kg) and SR were injected without PNZ. **D:** Bic (1.5 mg/kg) and SR were injected 30 min after PNZ (0.2 mg/kg) and FMZ (5 mg/kg). Doses of SR are symbol-coded. Asterisks denote significance of difference from "no SR" values. Data normalized to vehicle control. * - $P<0.05$, n=6-7, Bonferroni post-hoc test.

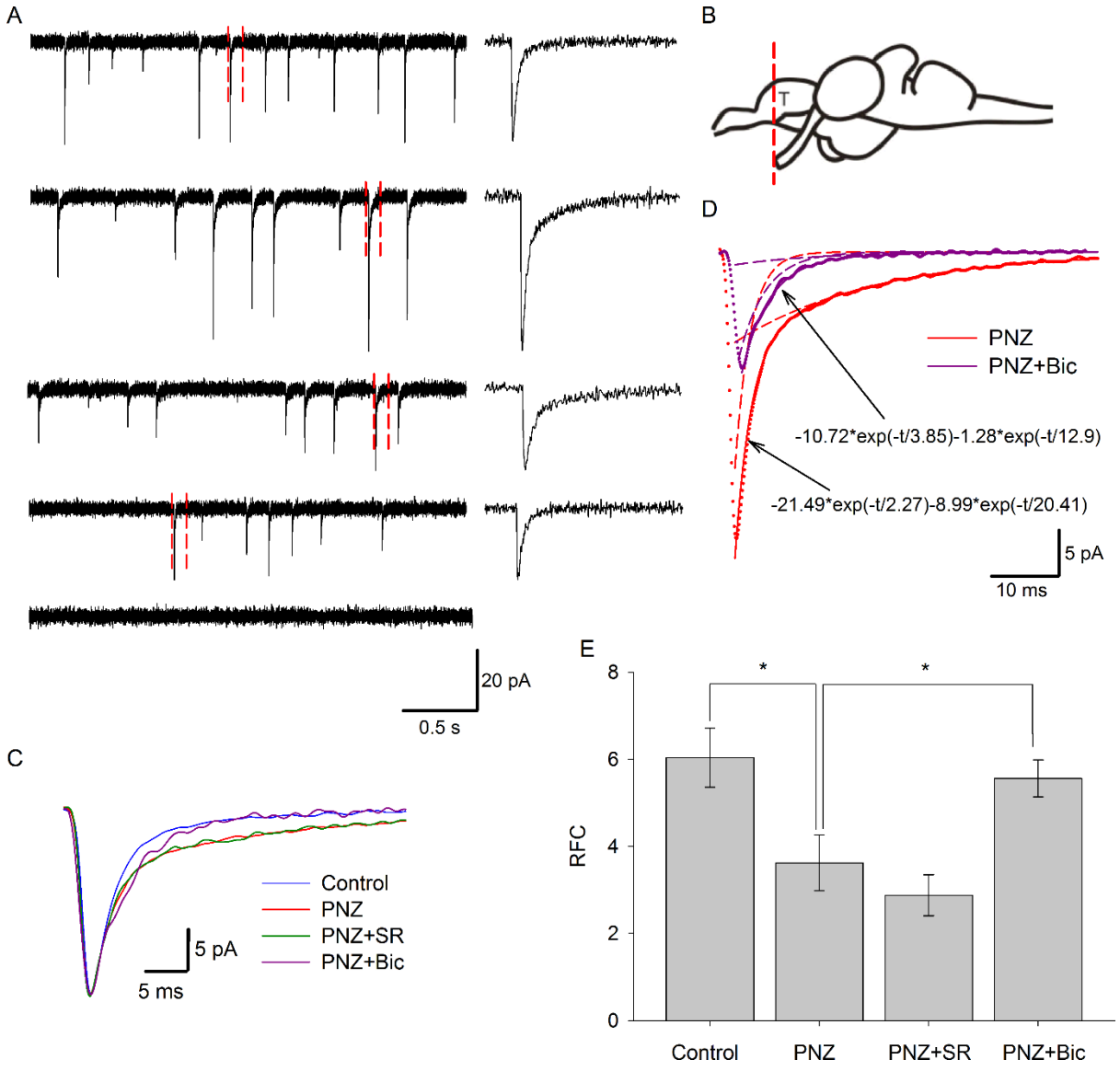

Figure 3. SR and Bic display different types of antagonism to GABA+PNZ effect on spontaneous IPSCs. A: Example traces of GABA-ergic spontaneous activity. From top to bottom: Control, PNZ, PNZ+SR, PNZ+Bic, PTX. Dashed lines denote example IPSCs expanded on the right. **B:** Sketch of *C. gibelio* brain. "T" marks telencephalon, dashed line shows approximate direction of slice cutting. **C:** Averaged spontaneous IPSCs normalized to peak amplitude of control event; note change of IPSC decay kinetics induced by Bic in contrast to SR. **D:** Analysis paradigm for response decay kinetics with bi-exponential fitting. Lines through data points: best-fit biexponential functions; the dashed lines of the same colour are plots of the fast and slow components alone. Ratio of fitting coefficients (RFC) of

fast (τ=2.27) to slow (τ=20.41) component generated under PNZ obtained as 21.49/8.99=2.39; application of Bic augmented decay components ratio to 10.72/1.38=8.38. **E:** Statistical summary on changes of RFC induced by GABAR ligands. * - P<0.05, n=6, paired Student's t-test.

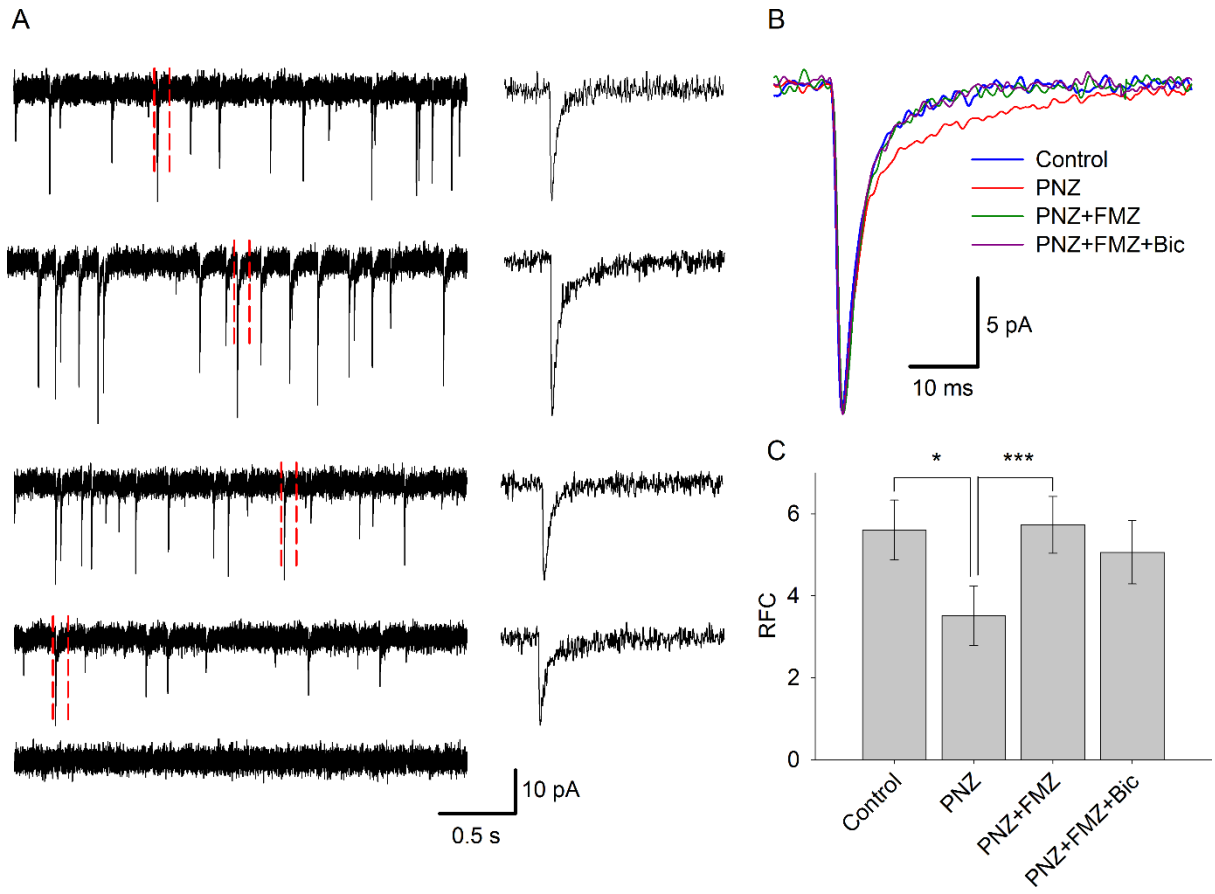

Figure 4. Pre-application of FMZ prevents effect of Bic on IPSC decay kinetics. A: Example traces of GABA-ergic spontaneous activity. From top to bottom: Control, PNZ, PNZ+FMZ, PNZ+FMZ+Bic, PNZ+FMZ+Bic+PTX. Dashed lines denote example IPSCs expanded on the right. **B:** Averaged spontaneous IPSCs normalized to peak amplitude of control event. **C:** Statistical summary on changes of RFC induced by GABAR ligands. * - $P<0.05$, *** - $P<0.001$, n=6, paired Student's t-test.

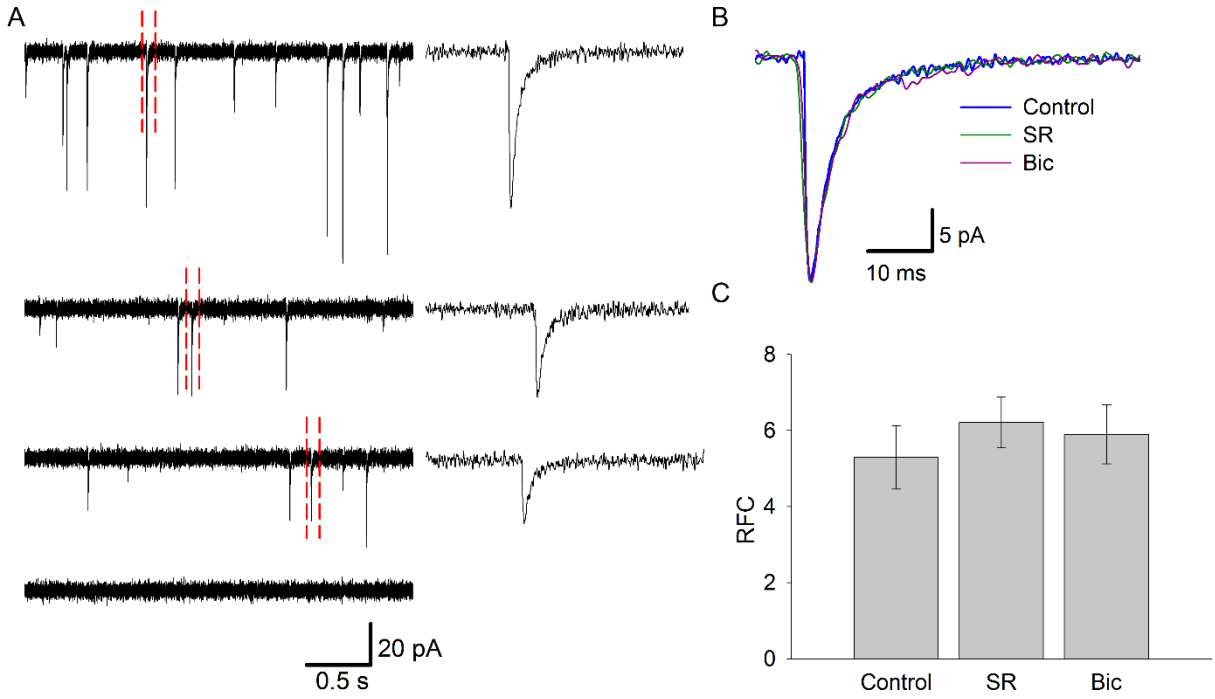

Figure 5. SR and Bic do not alter response kinetics generated by endogenous GABA. A: Example traces of GABA-ergic spontaneous activity. From top to bottom: Control, SR, Bic, PTX. Dashed lines denote example IPSCs expanded on the right. **B:** Averaged spontaneous IPSCs normalized to peak amplitude of control event; no change of response decay kinetics observed. **C:** Statistical summary on changes of RFC induced by GABAR ligands.

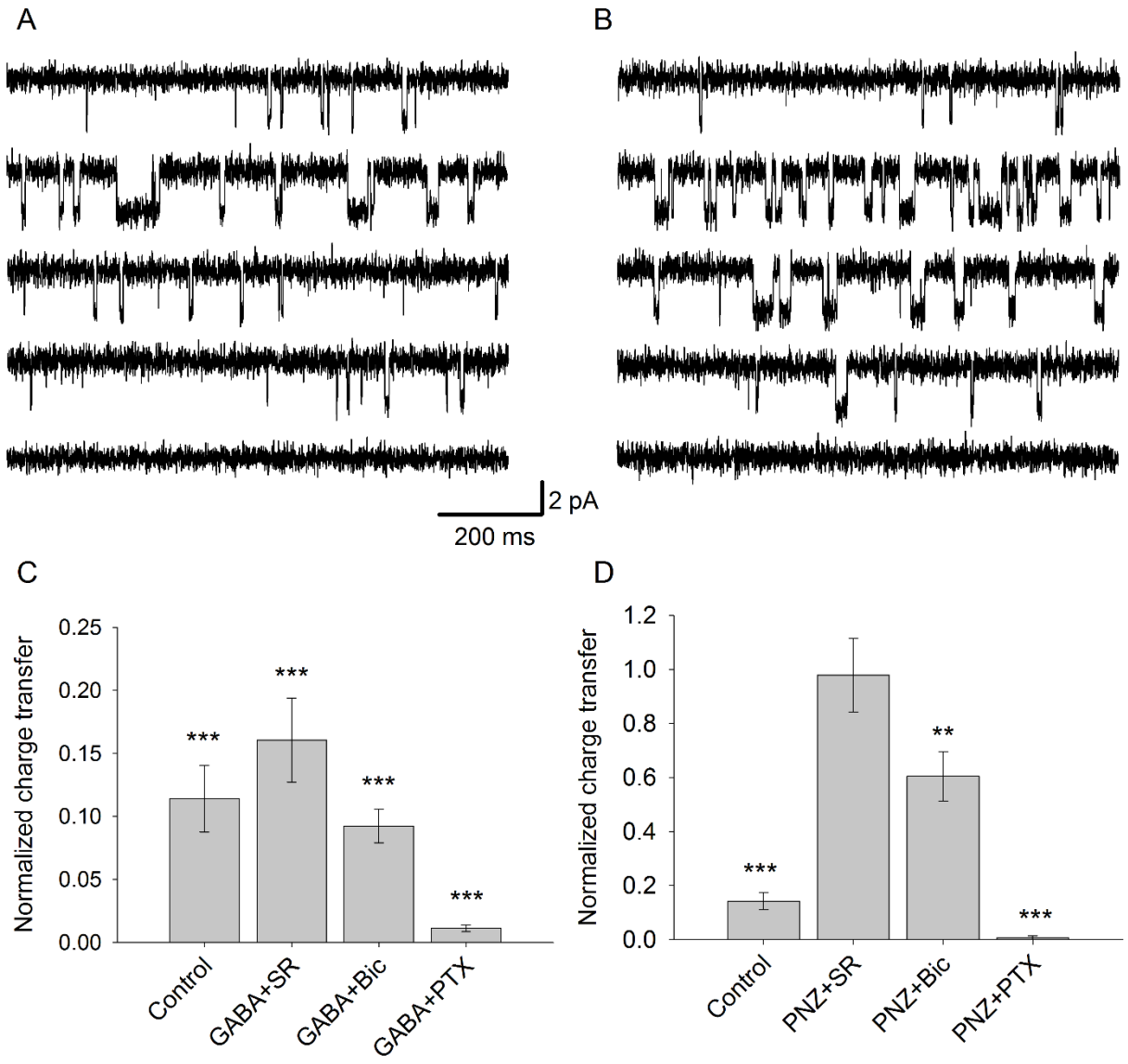

Figure 6. Bic, but not SR, antagonizes PNZ effect on single-channel GABAR openings. A: Example traces, channel openings induced by GABA. From top to bottom: Control (spontaneous openings), GABA, GABA+SR, GABA+Bic, GABA+PTX. **B:** Example traces, channel openings induced by PNZ. From top to bottom: Control, PNZ, PNZ+SR, PNZ+Bic, PNZ+PTX. Scale bars apply to A and B. **C:** Statistical summary of A. Charge transfer per second normalized to value generated by application of GABA; SR and Bic suppress GABA effect. **D:** Statistical summary of B. Charge transfer per second normalized to value generated by application of PNZ. Bic, but not SR, suppresses effect of PNZ. Asterisks indicate significance of difference from unity. ** - P<0.1, *** - P<0.01, n=6, paired Student's t-test.

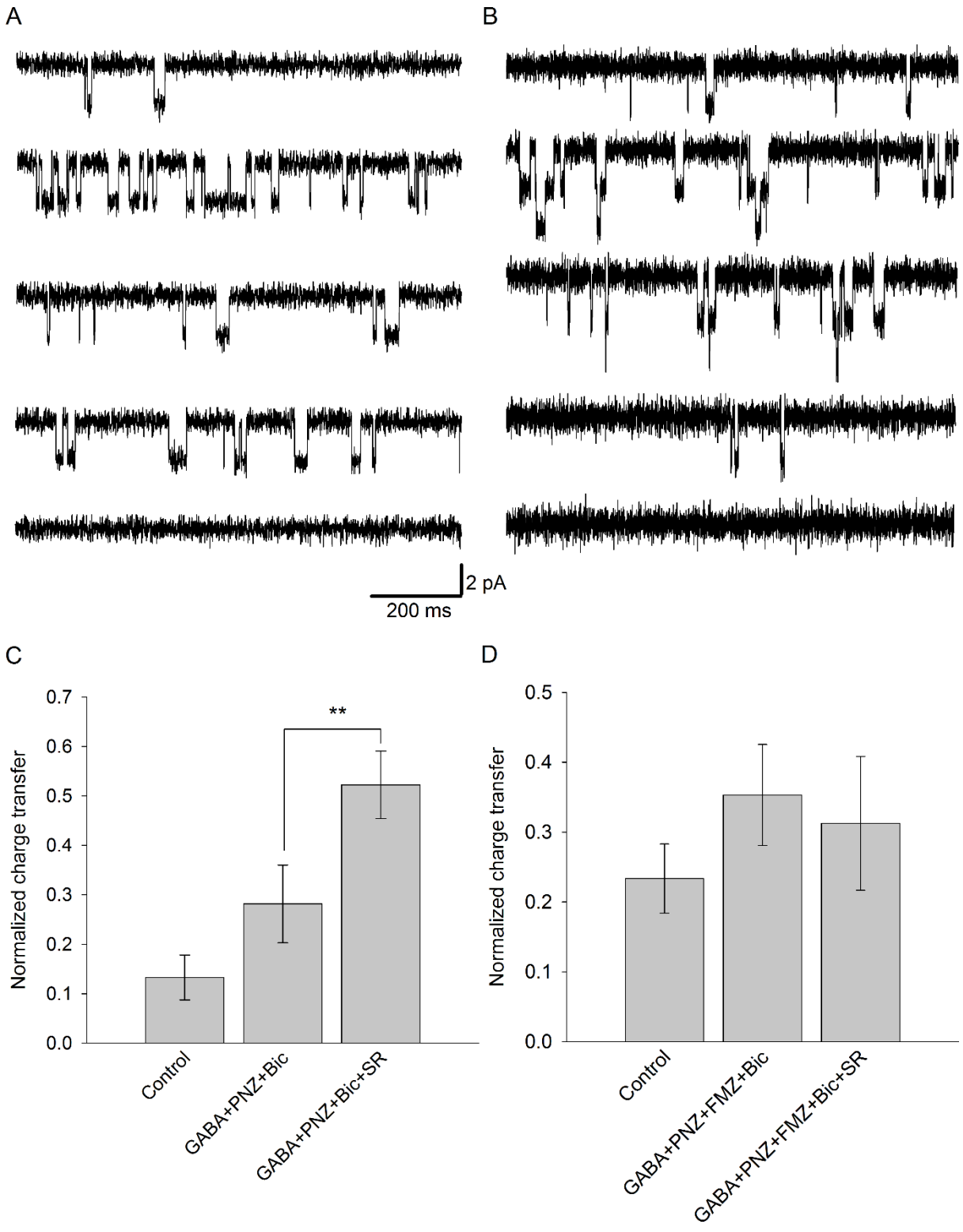

Figure 7. FMZ prevents conversion of Bic effect by SR on single-channel openings.

A: Traces from top to bottom: Control (no ligands), GABA+PNZ, GABA+PNZ+Bic,

GABA+PNZ+Bic+SR, GABA+PNZ+Bic+SR+PTX. **B:** Traces from top to bottom: Control, GABA+PNZ, GABA+PNZ+FMZ+Bic, GABA+PNZ+FMZ+Bic+SR, GABA+PNZ+FMZ+Bic+SR+PTX. Scale bars apply to A and B. **C:** Statistical summary for A. **D:** Statistical summary for B. In C and D data on charge transfer normalized to value generated by GABA+PNZ. ** - $P<0.01$, n=6, paired Student's t-test.