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ARTICLE

Voltage-clamp evidence of $GABA_A$ receptor subunit-specific effects: pharmacodynamic fingerprint of chlornordiazepam, the major active metabolite of mexazolam, as compared to alprazolam, bromazepam, and zolpidem

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Abstract

Background Anxiolytic benzodiazepines, due to their clinical effectiveness, are one of the most prescribed drugs worldwide, despite being associated with sedative effects and impaired psychomotor and cognitive performance. Not every $GABA_A$ receptor functions in the same manner. Those containing α1 subunits are associated with sleep regulation and have a greater effect on the sedative-hypnotic benzodiazepines, whereas those containing α2 and/or α3 subunits are associated with anxiety phenomena and have a greater effect on the anxiolytic benzodiazepines. Therefore, characterization of the selectivity profile of anxiolytic drugs could translate into a significant clinical impact.

Methods The present study pharmacodynamically evaluated chlornordiazepam, the main active metabolite of mexazolam, upon $GABA_A$ receptors containing α2 and/or α3, anxiety-related, and those containing an α1 subunit, associated with sleep modulation.

Results As shown by whole-cell patch-clamp data, chlornordiazepam potentiated GABA-evoked current amplitude in α2 and α3 containing receptors without changing the current amplitude in α1 containing receptors. However, current decay time increased, particularly in $GABA_A$ receptors containing α1 subunits. In contrast, other anxiolytic benzodiazepines such as alprazolam, bromazepam, and zolpidem, all increased currents associated with $GABA_A$ receptors containing the α1 subunit.

Conclusions This novel evidence demonstrates that mexazolam (through its main metabolite chlornordiazepam) has a "pharmacodynamic fingerprint" that correlates better with an anxiolytic profile and fewer sedative effects, when compared to alprazolam, bromazepam and zolpidem, explaining clinical trial outcomes with these drugs. This also highlights the relevance of the pharmacological selectivity over $GABA_A$ receptor subtypes in the selection of benzodiazepines, in addition to their clinical performance and pharmacokinetic characteristics.

Keywords Pharmacodynamic fingerprint · Mexazolam · Chlornordiazepam · Alprazolam · Bromazepam · $GABA_A$ receptors

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Abbreviations

ALZ	Alprazolam
BRO	Bromazepam
CND	Chlornordiazepam
$GABA_A$	Gamma-aminobutyric acid A
MEX	Mexazolam
ZLP	Zolpidem

Introduction

Gamma-aminobutyric acid A ($GABA_A$) receptors are anion channels selective for chloride which are phasically or tonically activated, leading to inhibition of nerve transmission in the perisynaptic and extrasynaptic sites, respectively [1, 2]. The $GABA_A$ receptor consists of 5 subunits that constitute a chloride channel and present different subunit compositions in several combinations. These subunits can have different isoforms, which in the case of the alpha subunit, α, are α1 to α6 [3, 4]. Not every $GABA_A$ receptor functions in the same way, and this is strongly dependent on subunit composition. Synaptic benzodiazepine-sensitive $GABA_A$ receptors are composed of two β subunits plus a γ subunit of either the γ2 or γ3, plus two α subunits, whilst benzodiazepine-insensitive $GABA_A$ receptors are composed of α4, α6, γ1, or δ subunits [1, 4]. Synaptic benzodiazepine-sensitive $GABA_A$ receptors mediate phasic inhibition. Those containing α1 subunits may be more relevant in regulating sleep and have a higher affinity for the sedative-hypnotic benzodiazepines [4]. Additionally, recent data suggest a relevant role of $GABA_A$ receptors containing α1 subunits in the mechanism of addiction and tolerance during benzodiazepine treatment [5, 6]. Those containing α2 and/or α3 subunits have been described to be more important in regulating anxiety and have a higher affinity for anxiolytic benzodiazepines [4]. In benzodiazepine-sensitive $GABA_A$ receptors, the neurotransmitter GABA, acting alone, increases the opening frequency of the chloride channel of the $GABA_A$ receptor to a limited extent. The allosteric modulation of the $GABA_A$ receptor by benzodiazepines has been shown to increase the opening frequency of the chloride channel to a higher extent than in the absence of this drug, leading to a more efficacious and faster hyperpolarization of the cell, consequently decreasing neuronal firing [1, 2]. $GABA_A$ receptors containing α5 subunits have a limited distribution in the brain, being mainly restricted to dendrites of hippocampal CA1 pyramidal cells, and have been associated with memory and learning processes [7, 8]. The α5 $GABA_A$ receptors were initially thought to be essentially present in extrasynaptic locations and to mediate a tonic inhibition of CA1 pyramidal cells. Presently, in addition to the extrasynaptic location, it is thought that these receptors also have a synaptic location [7–10]. 1Benzodiazepine-insensitive $GABA_A$ receptors containing α4 and α6 subunits are located extrasynaptically and mediate tonic inhibition [1]. Currently, there are no selective benzodiazepines for $GABA_A$ receptors with different subunit compositions, although several attempts were made to identify such compounds [11–13]. In particular, there has been an effort to discover and develop selective α2/3 subunits compounds, and currently, there are two such compounds in clinical development [14, 15].

Mexazolam (MEX), also known as CS-386, is an anxiolytic oxazolo-benzodiazepine (Fig. 1) indicated for the management of anxiety disorders whether associated or not with psychoneurotic conditions and is currently marketed in 29 countries, mainly in Europe, Africa, and Latin America [16]. Bromazepam (BRO) and alprazolam (ALZ), both triazolobenzodiazepines, are widely used as anxiolytic benzodiazepines that have the most recent head-to-head studies with MEX [16].

There is clinical evidence suggesting that MEX has reduced effects on psychomotor and cognitive performance [16–19], which is not the case with other benzodiazepines, such as ALZ and BRO [20–23]. Regarding efficacy and tolerability, there are four double-blind randomized trials directly comparing MEX, ALZ, and BRO: two trials comparing ALZ and BRO [24, 25], one comparing MEX and BRO [26], and another comparing MEX and ALZ [27] MEX showed a greater anxiolytic effect than BRO as assessed by the Hamilton anxiety scale (HAM-A). The other three studies did not show statistically significant effects on HAM-A. Following oral administration of the parent drug, MEX is transferred into the liver at a high concentration and due to a fast first-passage effect, is not detected in blood; only its active metabolites are found, being chlornordiazepam (CND), the main plasmatic metabolite [16, 28]. Additionally, no central nervous system distribution data are available for MEX and, therefore, although very unlikely, it is not possible to rule out some brain distribution of the parent molecule. BRO is indicated for the management of anxiety, tension and other somatic or psychiatric complaints associated with anxiety [29], and ALZ is indicated in anxiety states and panic-associated disorders [30, 31]. The metabolites of BRO and ALZ are less active and have much lower plasma concentration than the parent drug, thereby suggesting that they have only a residual contribution to the clinical effect [29, 32].

The purpose of this study was to evaluate if CND, the main active metabolite of MEX, might have a preferential affinity for α2 and α3 $GABA_A$-containing receptors when compared to α1 $GABA_A$-containing receptors. For this purpose, the affinity of MEX and its main metabolite CND to different synaptic $GABA_A$ receptor subtypes was assessed. The affinity of ALZ, BRO and zolpidem (ZLP) to different synaptic $GABA_A$ receptor subtypes was also evaluated. This is the first study demonstrating that the effects of CND upon

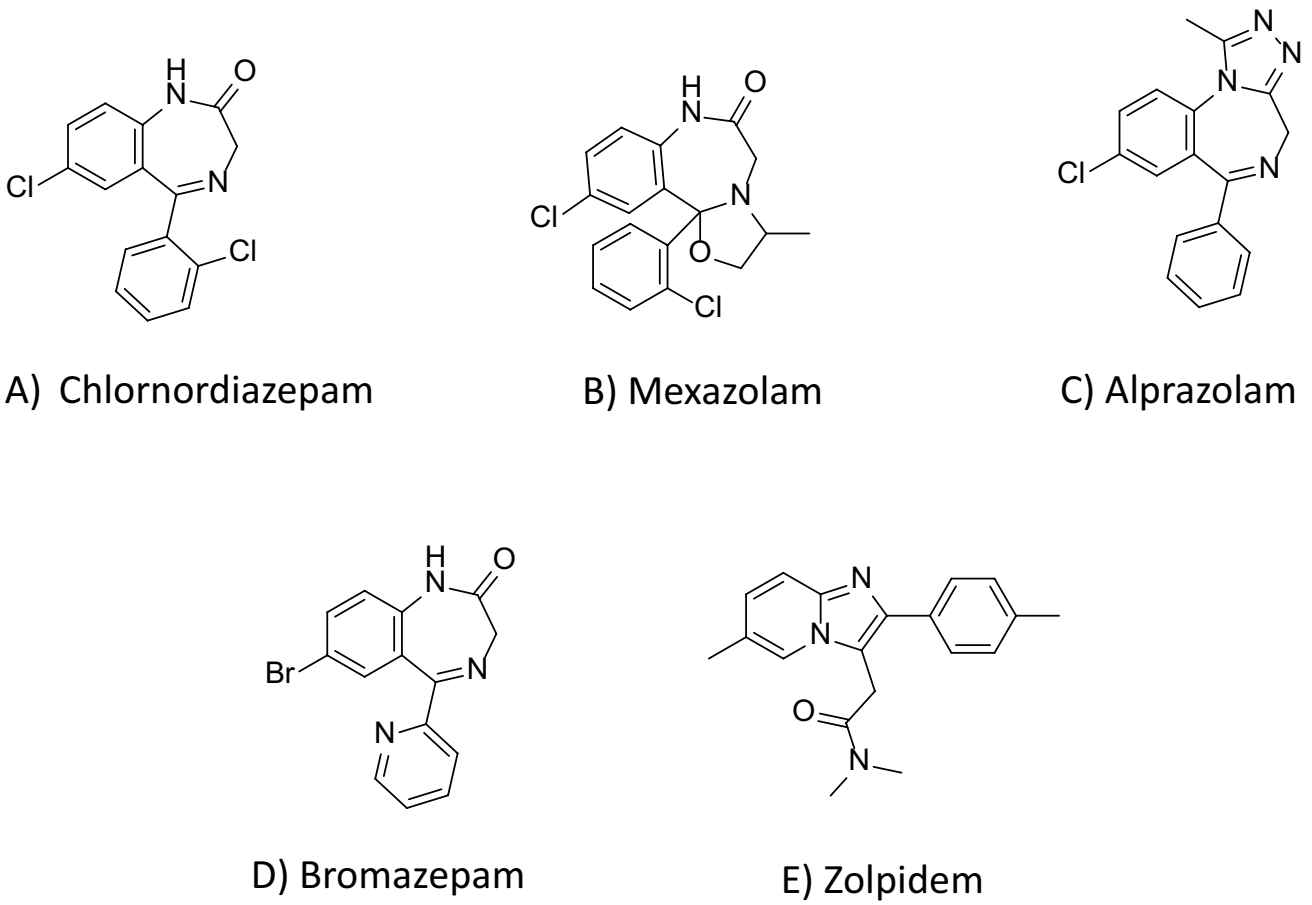


Fig. 1 Chemical structure of the different compounds. **A** chlornordiazepam (CND), **B** mexazolam (MEX), **C** alprazolam (ALZ), **D** bromazepam (BRO), **E** zolpidem (ZLP)

GABA currents, in contrast to all other tested compounds, are mediated mainly through α2 and α3 $GABA_A$-containing receptors and devoid of effects on the current amplitude of α1 containing $GABA_A$ receptors. It is suggested that such selectivity may explain the low incidence of mexazolam effects on psychomotor performance [18, 19].

Materials and methods

Test systems

Manual whole-cell patch-clamp experiments were performed in mouse fibroblasts cells Ltk-11, (ATCC Catalog no CRL-10422, BSYS, Switzerland) stably expressing human $GABA_A$-receptors with the following subunit composition: α1β2γ2, α2β2γ2, α3β2γ2 or α5β2γ2. The cells were divided at a confluence of about 50–80% and kept at 37 °C in a humidified atmosphere with 5% CO_2 (relative humidity of about 95%). The cells were continuously maintained and passaged in sterile culture flasks containing a 1:1 mixture of Dulbecco's modified eagle medium (DMEM) and nutrient mixture D-MEM/F-12 1x (Sigma-Aldrich, St. Louis, MO) liquid with L-glutamine supplemented with 10% fetal bovine serum and 1.0% penicillin/streptomycin solution (GIBCO™; Zug, Switzerland). The complete medium was supplemented with the antibiotic Geneticin (GIBCO, Sigma; α1β2γ2 and α2β2γ2: 500 µg/mL, α3β2γ2: 250 µg/mL, α5β2γ2: 100 µg/mL). The cells were seeded in 35 mm culture dishes at a density that allowed single cells to be recorded.

Equipment and whole-cell patch clamp recordings

The equipment used was an Amplifier EPC-10 (HEKA Electronics; Germany), a Headstage Preamplifier EPC-10 (HEKA Electronics; Germany), and the Software Patch-Master (HEKA Electronics; Germany). The bath solution included the following components: sodium chloride 137 mM, potassium chloride 4 mM, calcium chloride 1.8 mM, magnesium chloride 1 mM, HEPES 10 mM (Huberlab; Switzerland), D-glucose 10 mM and pH (NaOH) 7.4. The intracellular solution included the following

components: potassium chloride 130 mM, magnesium chloride 1 mM Mg-ATP 5 mM, HEPES 10 mM (Huberlab; Switzerland), EGTA 5 mM and pH (KOH) 7.2. During experiments, cells were continuously superfused using a custom-built fast application system with bath solution at room temperature (1.5–1.9 mL/min, 19–30 °C). Whole-cell patch-clamp recordings were carried out with the aid of an inverted microscope (Zeiss, Germany) and glass micropipettes (2.5–6.0 MΩ) that were manually driven by a micromanipulator (PatchStar, Scientifica, UK). After obtaining a gigaohm seal (> 1 GΩ), the membrane voltage was clamped at a holding potential of − 80 mV. Currents were elicited by transient application of GABA (gamma-aminobutyric acid, 5 µM) and their modulation by different test compounds was assessed. 5 µM GABA was used for all GABA receptor subtypes as this was close to the EC50 value for all of the assays (B'SYS, personal communication). After a stable baseline in response to GABA applications was achieved, increasing cumulative concentrations of a test item were applied to each cell recorded from. GABA or GABA containing a concentration of the test item was applied for 4 s, between two GABA applications bath solution or bath solution containing a corresponding concentration of test item, was perfused for 30 s, each concentration was applied 3 times. For the time-matched vehicle control experiments, GABA was applied in the presence of 0.1% DMSO (Vehicle control), after a stable baseline was achieved. At the end of the experiments, the GABA-A receptor antagonist bicuculine (10 µM) was applied as a positive control [33]. At least $n = 3$ cells were tested for each condition. Test compounds were used at the following concentrations: CND (1.0 nM, 4.0 nM, 10 nM, 40 nM and 100 nM), ALZ (10 nM, 40 nM, 100 nM, 400 nM and 1000 nM), MEX (10 nM, 40 nM, 100 nM, 400 nM and 1000 nM), ZLP (10 nM, 40 nM, 100 nM, 400 nM and 1000 nM) and BRO (40 nM, 100 nM, 400 nM, 1000 nM and 4000 nM). Concentrations were selected to provide a compound-specific dose-dependent response for current amplitude sufficient to unveil $GABA_A$ subunit selectivity with the compound having effects in at least one of the subunits. For all subunits where effects are observed at the lowest concentration, there is no effect of the compound. The tested concentrations also cover the reported Cmax data for all compounds (in ng/mL): ALZ—12 to 22 [34]; BRO—72 [35]; MEX—6.8 to 10.2 of CND [36]; ZLP—59 to 121 [37]. Compound concentrations tested in ng/mL were in the following range: ALZ (3.1–309.0), BRO (12.6–1264.6), CND (0.3–30.5), MEX (3.6–363.2), ZLP (3.1–307.4).

Currents induced by the application of GABA (5.0 µM) were measured before the application of allosteric modulators. Cells were only included when (i) the seal resistance remained above 300 MΩ throughout the experiment, (ii) GABA (5.0 µM) peak current amplitude stayed between 0.5 and 2 nA, and (iii) currents varied only 15% along 3 consecutive applications of GABA performed before drug testing Nonetheless, data also included 4 cells whose results matched with other cells from the respective data sets, even though the current amplitude was 10% outside the criteria.

Drugs

Salts in recording solutions were obtained from Sigma-Aldrich, St. Louis, MO. The agonist item was GABA (gamma-Aminobutyric acid), the test items were chlornordiazepam [7-chloro-5-(2-chlorophenyl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one] also known as chlordesmethyldiazepam, alprazolam [8-chloro-1-methyl-6-phenyl-4H-benzo[f][1,2,4]triazolo[4,3-a][1,4]diazepine], bromazepam [7-bromo-5-(pyridin-2-yl)-1,3-dihydro-2H-benzo[e][1,4]diazepin-2-one], mexazolam [10-chloro-11b-(2-chlorophenyl)-3-methyl-2,3,7,11b-tetrahydrobenzo[f] oxazolo[3,2-d][1, 4] diazepin-6(5H)-one)] (BIAL, Portugal) and zolpidem [N,N-dimethyl-2-(6-methyl-2-(p-tolyl) imidazo[1,2-a]pyridin-3-yl)acetamide], the reference compound was bicuculline, (6R)-6-[(5S)-6-methyl-7,8-dihydro-5H-[1,3]dioxolo[4,5-g]isoquinolin-5-yl]-6H-furo[3,4-g][1,3] benzodioxol-8-one and vehicle was DMSO. A 20 mM stock of GABA was prepared in ddH2O and kept frozen, and bicuculline was prepared as a 10 mM stock in DMSO and kept frozen. CND: 100 µg/mL stock in acetonitrile, alprazolam, bromazepam, zolpidem: 1 mg/mL stocks in methanol, zolpidem: 10 mM stock in DMSO. All test item stock solutions were kept frozen (− 10 to – 30 °C).

Data analysis

For each cell, GABA-evoked currents (i) in presence of the allosteric modulator (AM) were converted to the percent value of GABA-evoked response in the absence of the modulator, i.e., i(GABA + AM)/iGABA × 100. Time-matched vehicle control experiments and bicuculline applications were treated accordingly. For each baseline and compound concentration, the current decay of the GABA response was fitted by the exponential equation $i(t) = \text{imax} * e^{(-t/\tau)}$ where imax is the maximal current of that cell, t is the time (s) and τ is the time constant of current decay (FitMaster software, HEKA Electronics). The time constant of the current decay (τ) was determined for each cell and all tested concentrations. Cells were $n = 3$–6 for every condition. Data were checked for normal distribution in SigmaPlot (Version 11.2.0.5) using the Shapiro–Wilk Normality test. All data sets were normally distributed. To determine statistical significance ($p < 0.05$), a one-way Analysis of variance (ANOVA) followed by a Dunnett's multiple comparison test was used (GraphPad Prism 5).

Results

The effect (%) of CND, the main active metabolite of MEX, was assessed in $GABA_A$ receptors with different subunit compositions. CND had an effect mostly on the current amplitude of $GABA_A$ receptors containing α2, $F_{4,3} = 23.55$, $p < 0.0001$ and α3, $F_{4,3} = 36.45$, $p < 0.0001$ with a small effect on α5, $F_{4,6} = 23.72$, $p < 0.0001$ and no effect on α1 (not statistically significant) (Fig. 2A; Table 1). The effect observed (%) upon $GABA_A$ current decay time was divergent from current amplitude, with CND having a major effect on α1 $GABA_A$-containing receptors, $F_{4,4} = 5.142$, $p = 0.0047$ and a less marked effect on α2, $F_{4,3} = 10.87$, $p = 0.0004$, α3, $F_{4,3} = 12.85$, $p = 0.0002$, and α5, $F_{4,6} = 28.62$, $p < 0.0001$ (Fig. 3A; Table 2). This suggests that CND's main effects are due to interactions with $GABA_A$ receptors containing α2 and α3 subunits that have been characterised as the main mediators of anxiolytic effects of benzodiazepines.

The parent compound MEX was also assessed in the same panel of $GABA_A$ receptors which served as a control. MEX had statistically significant effects (%) in α1 GABA-containing receptors, $F_{4,4} = 19.12$, $p < 0.0001$. There was an interesting effect (%) on low compound concentrations with an inhibitory effect on α2, $F_{4,5} = 7.161$, $p = 0.0004$ and α5 mediated currents, $F_{4,3} = 19.28$, $p < 0.0001$, whose biological relevance is difficult to perceive. No effect was observed for α3 $GABA_A$-containing receptors (not statistically significant) (Fig. 2B; Table 1). Regarding current decay time, a statistically significant effect (increase) was observed in α1 $GABA_A$ containing receptors $F_{4,4} = 5.958$, $p = 0.0023$ and α5 $F_{4,3} = 4.299$, $p = 0.0179$, with no statistically significant effect observed in α2 and α3 (Fig. 3B; Table 2). This data indicates that MEX itself has an effect mostly mediated by $GABA_A$ receptors containing α1 units, though this might have low biological relevance as the parent compound is not detected in circulation.

To be able to confirm that the clinical advantage of CND could be translated from *in-vitro* data, other relevant and widely used benzodiazepines, ALZ and BRO, were also tested, including ZLP, a reference sleep inducer. ALZ had an effect (%) only on α1 $GABA_A$-containing receptors $F_{4,3} = 3{,}225$, $p = 0.0448$ and α3, $F_{4,3} = 12.79$, $p = 0.0002$, with no effect in α2 and α5 (no statistically significant results) (Fig. 2C; Table 1). Regarding current decay time, there was no difference between α1, $F_{4,3} = 4.663$, $p = 0.0135$, and α3 containing receptors effects (%), $F_{4,3} = 4.407$, $p = 0.0164$, with no statistically significant effect observed in α2 containing receptors, the effect on α5 $GABA_A$-containing receptors was less pronounced than other subunits, with no statistically significant effect (Fig. 3C; Table 2).

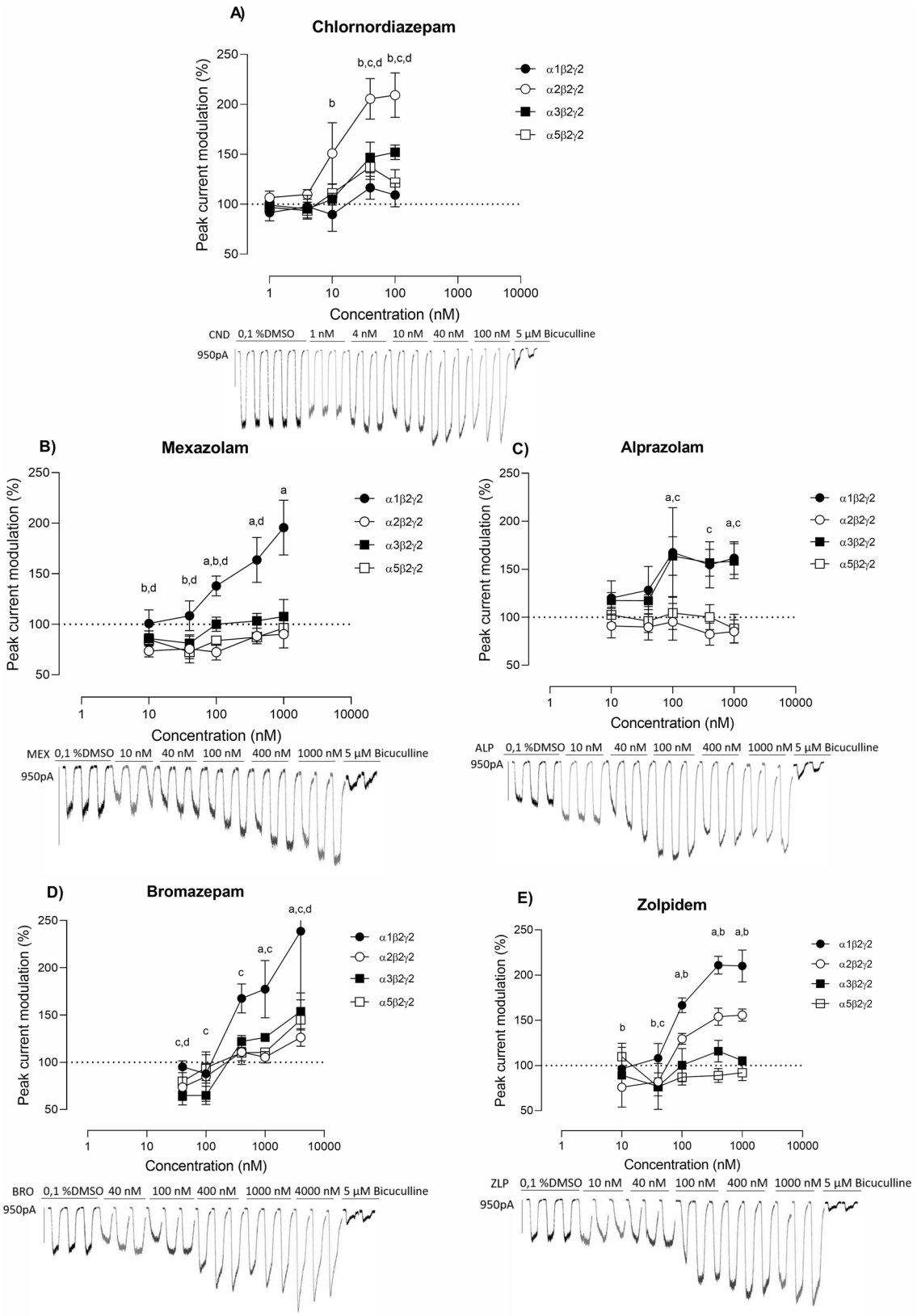
BRO showed effect (%) upon $GABA_A$ receptors containing α1, $F_{4,4} = 12.15$, $p < 0.0001$ and α3, $F_{4,4-6} = 52.14$, $p < 0.0001$ subunits but to a lesser extent in $GABA_A$ receptors containing the α5 subunit, $F_{4,3} = 33.78$, $p < 0.0001$. Effects on $GABA_A$ receptors containing the α2 subunit did not reach statistical significance (Fig. 2D; Table 1). Regarding current decay time, the data was highly variable; however, an effect (%) in $GABA_A$ receptors containing the α3 subunit $F_{4,4-6} = 5.222$, $p = 0.0032$, and to a lesser extent in the α5 subunit, $F_{4,3} = 2.663$, $p = 0.0764$, was statistically significant, compared to control (Fig. 3D; Table 2). The effects observed on current amplitude indicate that bromazepam´s anxiolytic effects are mainly mediated by $GABA_A$ receptors containing the α3 subunit accompanied by α1 potentiation.

ZLP, had a statistically significant effect (%) on the current amplitude of $GABA_A$ receptors containing α1 $F_{4,3} = 69.82$, $p < 0.0001$ and α2 subunits $F_{4,3} = 120.8$, $p < 0.0001$, which were higher in α1. No effect was observed in the other $GABA_A$ receptors tested. (Fig. 2E; Table 1). Effects (%) of ZLP on current decay time were observed for all GABA receptors tested, with the highest effect being observed for $GABA_A$ receptors containing the α3 subunit $F_{4,3} = 8.059$, $p = 0.0015$ (Fig. 3E; Table 2). This data indicates that ZLP increased inhibition is mediated by $GABA_A$ receptors containing α1 (mainly) and α2 subunits.

Importantly, among all the compounds tested, only CND was devoid of an effect statistically different from respective baseline control, at the highest concentration tested, on $GABA_A$ receptors containing α1 subunits and had a significant effect simultaneous on both $GABA_A$ receptors containing α2 and α3 subunits (Table 3).

Discussion

The main observation of this study is that CND does not modulate the current amplitude of $GABA_A$ receptors containing the α1 subunit, which has been strongly associated with sedative effects. The absence of an α1 effect is aligned with the preclinical and clinical evidence favouring MEX, or more accurately it is active metabolite CND since MEX is undetected in blood and appears to have a low propensity for sedative effects and reduced effects on psychomotor performance in vivo [16–19, 26, 27, 38]. Regarding psychomotor performance, two double-blind randomized clinical trials were conducted for MEX versus placebo, one in healthy volunteers and the other in patients with generalized anxiety disorder. Both studies concluded that MEX had reduced effects on psychomotor performance [18, 19]. On the other hand, a preclinical comparative electrophysiological study and double-blind clinical data have shown that ALZ induces sedation and impairs psychomotor performance [21, 23, 39, 40]. This is in line with the finding that alprazolam acts upon $GABA_A$ receptors containing the α1 subunit, as


A)
Chlornordiazepam
Peak current modulation (%)
Concentration (nM)
α1β2γ2
α2β2γ2
α3β2γ2
α5β2γ2
CND 0,1 %DMSO 1 nM 4 nM 10 nM 40 nM 100 nM 5 μM Bicuculline
950pA
B)
Mexazolam
MEX 0,1 %DMSO 10 nM 40 nM 100 nM 400 nM 1000 nM 5 μM Bicuculline
C)
Alprazolam
ALP 0,1 %DMSO 10 nM 40 nM 100 nM 400 nM 1000 nM 5 μM Bicuculline
D)
Bromazepam
BRO 0,1 %DMSO 40 nM 100 nM 400 nM 1000 nM 4000 nM 5 μM Bicuculline
E)
Zolpidem
ZLP 0,1 %DMSO 10 nM 40 nM 100 nM 400 nM 1000 nM 5 μM Bicuculline

◂**Fig. 2** Effect of the different compounds—CND, MEX, ALZ, BRO and ZLP—on the peak current amplitude (mean ± SD) of $GABA_A$ receptors with different subunit compositions—α1β2γ2, α2β2γ2, α3β2γ2 or α5β2γ2. Percent of effect on current amplitude for the different compound concentrations was determined in relation to baseline control (perfusion of 0.1% DMSO) for each individual cell. **A** chlornordiazepam (CND), **B** mexazolam (MEX), **C** alprazolam (ALZ), **D** bromazepam (BRO), **E** zolpidem (ZLP). These experiments were performed in the presence of GABA. Representative tracings are for the effects of each compound on α1β2γ2 $GABA_A$ receptors. Closed circles represent α1β2γ2, open circles represent α2β2γ2, closed squares represent α3β2γ2 and open squares represent α5β2γ2. A one-way ANOVA followed by Dunnett's multiple comparison test was used to compare each concentration with baseline control, $p < 0.05$ ($n = 3$–6); [a]statistically different from control for α1β2γ2; [b]statistically different from control for α2β2γ2, [c]statistically different from control for α3β2γ2; [d]statistically different from control for α5β2γ2

demonstrated here. BRO, which also acts upon $GABA_A$ receptors containing an α1 subunit, has also been reported, in double-blind clinical trials, to trigger motor impairment and promote altered performance during psychomotor performance tests [20, 22, 41].

A potential advantage of CND, over BRO and ALZ, is the modulation of both $GABA_A$ receptors containing α2 and α3 subunits, which are believed to mediate anxiolytic effects [4]. CND had an effect on the amplitude of $GABA_A$ currents mostly in receptors containing the α2 and α3 subunits, whereas ALZ and BRO had an effect only on $GABA_A$ receptors containing the α3 subunit, with no effect in α2 containing receptors. The fact that CND targets both $GABA_A$ receptors containing α2 and α3 subunits may translate into a more effective anxiolytic action when compared to targeting $GABA_A$ receptors containing α2 or α3 subunits alone since CND can target a wider variety/number of receptors. This enhanced anxiolytic effect of mexazolam versus other benzodiazepines, is supported by two double-blind randomized clinical trials [26, 27]. A double-blind randomized clinical trial comparing MEX and ALZ in generalized anxiety patients, showed a higher absolute rate of responders in the MEX group, although there were no statistically significant differences in the between-group comparisons, 80 vs 70% in HAM-A and 96.7 vs 86.7% for the clinical global impression (CGI) assessments [27]. Additionally, the fact that the incidence of adverse events was also higher in patients treated with ALZ [16, 27], is in line with the findings concerning the selectivity of effects upon $GABA_A$ receptors containing α1 subunits. Another double-blind randomized clinical trial comparing MEX and BRO in patients with anxiety showed that the reduction on the HAM-A scale was greater in patients treated with MEX than in patients treated with BRO, an improvement that was statistically significant [16, 26]. The results obtained with α1 and α3 $GABA_A$-containing receptors with ALZ versus BRO are congruent with clinical trials results. Two studies compared ALZ versus BRO [24, 25] in terms of efficacy (HAM-A scale) and tolerability (general side effects). In both studies, the efficacy and tolerability were better with ALZ, although in both domains of both studies, results were not statistically significant. Only for the adverse events drowsiness and rigidity, one of the studies did achieve statistical significance favouring ALZ [25]. Regarding the absence of an effect of ALZ on $GABA_A$ receptors containing an α2 subunit, it might be explained by findings that the potentiation of $GABA_A$ receptors containing an α3 subunit is adequate to produce the anxiolytic effect of benzodiazepines, even without potentiation through $GABA_A$ receptors containing the α2 subunit [42]. The same explanation might also apply to BRO.

The effect of $GABA_A$ receptors containing an α5 subunit has been the subject of debate, but it appears to correlate with memory [7]. CND and BRO had a small effect and ALZ had no effect on $GABA_A$ currents mediated by receptors containing an α5 subunit. In a head-to-head double-blind randomized clinical trial in patients with anxiety, memory changes were assessed using the digit span test and a questionnaire on memory retention and recall. The results demonstrated that MEX and BRO had improved the outcome on the memory test, since both had statistically significant improvement compared to baseline, due to the reduced anxiety; in addition, mexazolam showed a statistically significant improvement versus bromazepam [16, 26]. Two additional studies, both double-blind randomized clinical trials in healthy volunteers, revealed no effect of MEX and BRO on cognition processes [19, 43]. In contrast, there is concordant information, showing that ALZ negatively impacts cognition tests [21, 23, 44]. It is suggested that the cognitive processes, and consequently the cognitive tests, might be influenced not just by $GABA_A$ receptors containing α5 but also α1 subunits. This possibility is supported by findings that $GABA_A$ receptors containing both α1 and α5 subunits can contribute to the clinical cognitive effect. As an example, ZLP, is a strong agonist of α1 receptor subtype as demonstrated in the present study, and produced memory and cognitive impairments [5].

Regarding the relationship between $GABA_A$-containing receptors subunit composition, which determines $GABA_A$ receptor kinetic properties, inhibitory postsynaptic currents, and decay time data is not totally clear [45–48]. Long-term accumulation of desensitized states can modulate the amplitude of the synaptic response during repetitive stimulation [46, 47]. By increasing the recovery time from activation of $GABA_A$ receptors containing α1 subunits, i.e. increasing time decay, CND may decrease the frequency of activation of this subtype of GABA receptors. This is congruent with the clinical information reported earlier. ALZ increases the decay time in α1, α2 and α3 $GABA_A$-containing receptors which may also decrease their frequency of activation. In other words, the effect on

Table 1 Observed effect (%) on peak current amplitude (mean ± SD) after application of the different compounds

	CND		MEX		ALP		BRO		ZLP	
$\alpha_1\beta_2\gamma_2$	1 nM	91.61 ± 8.20 ($n=4$)	10 nM	100.69 ± 13.53 ($n=4$)	10 nM	119.73 ± 17.94 ($n=3$)	40 nM	95.27 ± 6.33 ($n=4$)	10 nM	96.40 ± 4.51 ($n=3$)
	4 nM	97.62 ± 11.30 ($n=4$)	40 nM	108.41 ± 14.73 ($n=4$)	40 nM	128.25 ± 24.74 ($n=3$)	100 nM	87.80 ± 9.96 ($n=4$)	40 nM	108.01 ± 16.31 ($n=3$)
	10 nM	89.65 ± 16.90 ($n=4$)	100 nM	137.98 ± 9.88 ($n=4$)*	100 nM	167.47 ± 46.45 ($n=3$)*	400 nM	167.58 ± 15.26 ($n=4$)	100 nM	166.71 ± 8.22 ($n=3$)*
	40 nM	116.48 ± 11.54 ($n=4$)	400 nM	163.58 ± 22.19 ($n=4$)*	400 nM	154.60 ± 23.96 ($n=3$)	1000 nM	177.37 ± 30.27 ($n=4$)*	400 nM	211.12 ± 9.81 ($n=3$)*
	100 nM	110.14 ± 11.87 ($n=4$)	1000 nM	195.54 ± 27.18 ($n=4$)*	1000 nM	161.55 ± 17.03 ($n=3$)*	4000 nM	238.78 ± 72.66 ($n=4$)*	1000 nM	210.20 ± 17.64 ($n=3$)*
$\alpha_2\beta_2\gamma_2$	1 nM	106.52 ± 6.70 ($n=3$)	10 nM	73.57 ± 6.04 ($n=5$)*	10 nM	90.96 ± 12.34 ($n=3$)	40 nM	73.69 ± 18.92 ($n=3$)	10 nM	75.99 ± 2.80 ($n=3$)*
	4 nM	109.81 ± 4.78 ($n=3$)	40 nM	75.59 ± 13.96 ($n=5$)*	40 nM	89.63 ± 13.50 ($n=3$)	100 nM	84.74 ± 26.29 ($n=3$)	40 nM	82.03 ± 2.48 ($n=3$)*
	10 nM	150.95 ± 30.66 ($n=3$)*	100 nM	72.44 ± 7.80 ($n=5$)*	100 nM	95.25 ± 19.24 ($n=3$)	400 nM	110.84 ± 13.17 ($n=3$)	100 nM	129.55 ± 5.97 ($n=3$)*
	40 nM	205.56 ± 20.33 ($n=3$)*	400 nM	88.28 ± 7.73 ($n=5$)	400 nM	82.44 ± 11.62 ($n=3$)	1000 nM	105.38 ± 6.03 ($n=3$)	400 nM	153.94 ± 9.47 ($n=3$)*
	100 nM	209.26 ± 22.31 ($n=3$)*	1000 nM	90.22 ± 13.71 ($n=4$)	1000 nM	85.11 ± 12.11 ($n=3$)	4000 nM	126.31 ± 9.30 ($n=3$)	1000 nM	155.78 ± 6.78 ($n=3$)*
$\alpha_3\beta_2\gamma_2$	1 nM	98.99 ± 1.74 ($n=3$)	10 nM	85.85 ± 7.54 ($n=3$)	10 nM	117.33 ± 8.29 ($n=3$)	40 nM	64.74 ± 5.23 ($n=6$)*	10 nM	89.26 ± 35.40 ($n=3$)
	4 nM	95.28 ± 1.34 ($n=3$)	40 nM	81.13 ± 7.06 ($n=3$)	40 nM	117.29 ± 6.22 ($n=3$)	100 nM	64.89 ± 9.63 ($n=6$)*	40 nM	76.88 ± 25.40 ($n=3$)
	10 nM	105.03 ± 5.97 ($n=3$)	100 nM	100.00 ± 7.05 ($n=3$)	100 nM	163.70 ± 20.15 ($n=3$)*	400 nM	122.01 ± 6.32 ($n=4$)*	100 nM	100.48 ± 18.49 ($n=3$)
	40 nM	146.76 ± 15.35 ($n=3$)*	400 nM	103.26 ± 7.47 ($n=3$)	400 nM	156.79 ± 13.93 ($n=3$)*	1000 nM	126.22 ± 3.03 ($n=4$)*	400 nM	115.83 ± 11.99 ($n=3$)
	100 nM	152.04 ± 7.34 ($n=3$)*	1000 nM	107.64 ± 16.93 ($n=3$)	1000 nM	158.43 ± 18.26 ($n=3$)*	4000 nM	153.86 ± 19.62 ($n=4$)*	1000 nM	105.53 ± 3.94 ($n=3$)
$\alpha_5\beta_2\gamma_2$	1 nM	95.86 ± 5.43 ($n=6$)	10 nM	84.85 ± 5.88 ($n=3$)*	10 nM	102.45 ± 8.14 ($n=4$)	40 nM	79.70 ± 1.89 ($n=3$)*	10 nM	109.69 ± 10.51 ($n=3$)
	4 nM	93.79 ± 7.65 ($n=6$)	40 nM	72.53 ± 6.84 ($n=3$)*	40 nM	95.93 ± 11.92 ($n=4$)	100 nM	94.23 ± 13.75 ($n=3$)	40 nM	76.35 ± 10.04 ($n=3$)*
	10 nM	111.25 ± 8.27 ($n=6$)	100 nM	83.71 ± 4.13 ($n=3$)*	100 nM	104.73 ± 17.17 ($n=4$)	400 nM	110.01 ± 8.49 ($n=3$)	100 nM	87.12 ± 8.75 ($n=3$)
	40 nM	135.94 ± 14.43 ($n=6$)*	400 nM	87.19 ± 2.67 ($n=3$)*	400 nM	100.07 ± 13.06 ($n=4$)	1000 nM	110.59 ± 2.06 ($n=3$)	400 nM	88.91 ± 7.74 ($n=3$)
	100 nM	122.77 ± 11.75 ($n=6$)*	1000 nM	96.11 ± 0.93 ($n=3$)	1000 nM	88.19 ± 14.88 ($n=4$)	4000 nM	144.95 ± 3.45 ($n=3$)*	1000 nM	91.95 ± 8.72 ($n=3$)

*Statistically different from respective baseline control using a one-way ANOVA followed by a Dunnett's multiple comparison test, $p<0.05$

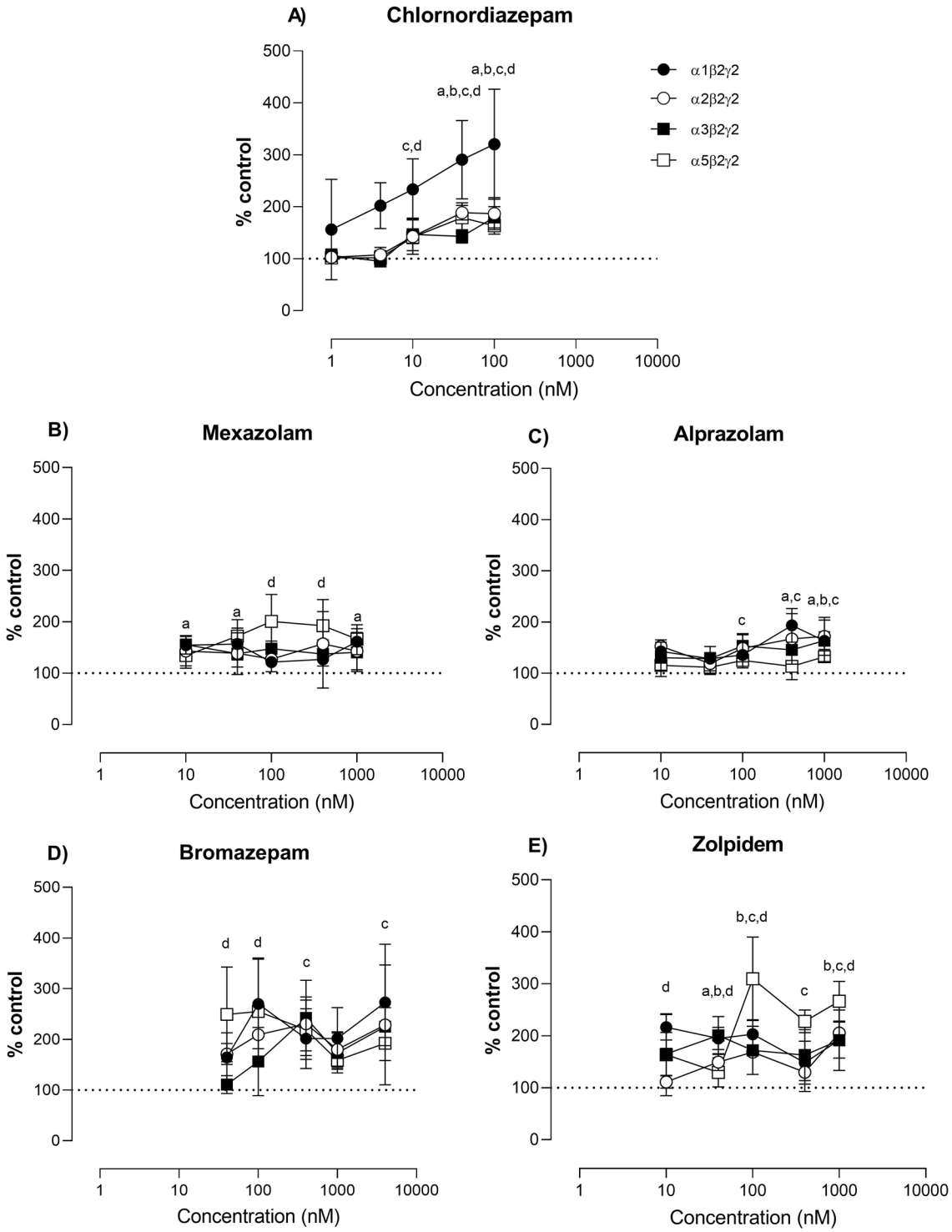
CND chlornordiazepam, *MEX* mexazolam, *ALZ* alprazolam, *BRO* bromazepam and *ZLP* zolpidem

Table 2 Observed effect (%) on current decay (mean ± SD) after application of the different compounds

	CND		MEX		ALP		BRO		ZLP	
$\alpha_1\beta_2\gamma_2$	1 nM	156.89 ± 98.17 (n = 4)	10 nM	154.70 ± 17.16 (n = 4)*	10 nM	142.06 ± 18.47 (n = 3)	40 nM	219.16 ± 101.25 (n = 4)	10 nM	165.10 ± 41.50 (n = 3)
	4 nM	202.65 ± 44.34 (n = 4)	40 nM	157.08 ± 31.23 (n = 4)*	40 nM	128.11 ± 11.29 (n = 3)	100 nM	271.02 ± 89.77 (n = 4)	40 nM	201.32 ± 36.78 (n = 3)*
	10 nM	240.25 ± 50.99 (n = 4)	100 nM	122.03 ± 5.00 (n = 4)	100 nM	135.49 ± 24.66 (n = 3)	400 nM	201.95 ± 58.61 (n = 4)	100 nM	172.26 ± 46.22 (n = 3)
	40 nM	291.72 ± 75.77 (n = 4)*	400 nM	126.92 ± 10.79 (n = 4)	400 nM	193.37 ± 33.41 (n = 3)*	1000 nM	202.54 ± 61.58 (n = 4)	400 nM	163.32 ± 48.53 (n = 3)
	100 nM	321.75 ± 106.06 (n = 4)*	1000 nM	163.39 ± 26.01 (n = 4)*	1000 nM	162.98 ± 41.41 (n = 3)*	4000 nM	274.14 ± 115.00 (n = 4)	1000 nM	192.17 ± 57.91 (n = 3)
$\alpha_2\beta_2\gamma_2$	1 nM	101.18 ± 7.03 (n = 3)	10 nM	142.80 ± 28.81 (n = 5)	10 nM	152.43 ± 11.73 (n = 3)	40 nM	171.24 ± 20.31 (n = 3)	10 nM	111.31 ± 12.03 (n = 3)
	4 nM	106.41 ± 15.29 (n = 3)	40 nM	138.69 ± 27.19 (n = 5)	40 nM	119.23 ± 21.30 (n = 3)	100 nM	208.83 ± 62.47 (n = 3)	40 nM	149.61 ± 15.52 (n = 3)*
	10 nM	141.38 ± 33.91 (n = 3)	100 nM	127.30 ± 24.36 (n = 5)	100 nM	148.47 ± 26.44 (n = 3)	400 nM	230.44 ± 53.35 (n = 3)	100 nM	168.79 ± 10.23 (n = 3)*
	40 nM	186.15 ± 15.92 (n = 3)*	400 nM	156.62 ± 85.23 (n = 5)	400 nM	166.63 ± 49.64 (n = 3)	1000 nM	179.61 ± 35.48 (n = 3)	400 nM	129.37 ± 36.75 (n = 3)
	100 nM	185.23 ± 32.82 (n = 3)*	1000 nM	143.10 ± 36.54 (n = 4)	1000 nM	171.41 ± 37.48 (n = 3)*	4000 nM	228.83 ± 118.59 (n = 3)	1000 nM	205.20 ± 20.54 (n = 3)*
$\alpha_3\beta_2\gamma_2$	1 nM	106.91 ± 11.57 (n = 3)	10 nM	155.40 ± 17.23 (n = 3)	10 nM	130.67 ± 23.67 (n = 3)	40 nM	114.98 ± 16.00 (n = 5)	10 nM	163.17 ± 77.87 (n = 3)
	4 nM	95.48 ± 5.97 (n = 3)	40 nM	137.52 ± 40.49 (n = 3)	40 nM	128.40 ± 23.78 (n = 3)	100 nM	156.39 ± 67.13 (n = 6)	40 nM	129.44 ± 27.69 (n = 3)
	10 nM	147.57 ± 30.53 (n = 3)*	100 nM	147.17 ± 14.37 (n = 3)	100 nM	153.48 ± 24.62 (n = 3)*	400 nM	242.41 ± 74.45 (n = 4)*	100 nM	309.47 ± 79.55 (n = 3)*
	40 nM	143.96 ± 11.72 (n = 3)*	400 nM	136.94 ± 23.78 (n = 3)	400 nM	145.29 ± 3.99 (n = 3)*	1000 nM	173.40 ± 39.49 (n = 4)	400 nM	228.07 ± 20.78 (n = 3)*
	100 nM	180.71 ± 18.91 (n = 3)*	1000 nM	139.90 ± 37.55 (n = 3)	1000 nM	163.62 ± 17.90 (n = 3)*	4000 nM	225.38 ± 37.80 (n = 4)*	1000 nM	266.62 ± 37.31 (n = 3)*
$\alpha_5\beta_2\gamma_2$	1 nM	101.10 ± 8.08 (n = 6)	10 nM	134.01 ± 24.95 (n = 3)	10 nM	115.54 ± 21.62 (n = 3)	40 nM	248.72 ± 92.38 (n = 3)*	10 nM	215.94 ± 25.01 (n = 3)*
	4 nM	100.97 ± 14.69 (n = 6)	40 nM	172.27 ± 32.46 (n = 3)	40 nM	111.46 ± 12.96 (n = 3)	100 nM	254.70 ± 105.36 (n = 3)*	40 nM	194.68 ± 22.13 (n = 3)*
	10 nM	148.17 ± 13.15 (n = 6)*	100 nM	200.35 ± 52.65 (n = 3)*	100 nM	125.31 ± 5.21 (n = 3)	400 nM	218.66 ± 59.26 (n = 3)	100 nM	202.78 ± 29.25 (n = 3)*
	40 nM	179.48 ± 29.68 (n = 6)*	400 nM	192.43 ± 27.46 (n = 3)*	400 nM	113.63 ± 26.06 (n = 3)	1000 nM	158.65 ± 17.31 (n = 3)	400 nM	148.08 ± 42.14 (n = 3)
	100 nM	162.76 ± 15.70 (n = 6)*	1000 nM	166.28 ± 27.88 (n = 3)	1000 nM	132.48 ± 5.85 (n = 3)	4000 nM	191.72 ± 5.91 (n = 3)	1000 nM	191.58 ± 35.94 (n = 3)*

*Statistically different from respective baseline control using a one-way ANOVA followed by a Dunnett's multiple comparison test, $p < 0.05$

CND chlornordiazepam, *MEX* mexazolam, *ALZ* alprazolam, *BRO* bromazepam and *ZLP* zolpidem



amplitude and time decay on α1 and α3 might have opposite directions, which may have clinical implications. This finding was also verified with another benzodiazepine, i.e. flurazepam, which prolonged decay time and increased the amplitude in $GABA_A$ receptors containing α1 subunits [48, 49].

There are some limitations to be acknowledged in this electrophysiological experiment. One limitation of this study was that only the main metabolites were assessed, and it would be a more comprehensive assessment of all metabolites were studied. Another limitation is related to the difference between this electrophysiological evaluation

◂**Fig. 3** Effect of the different compounds—CND, MEX, ALZ, BRO and ZLP—on current decay time (Tau) (mean ± SD) of $GABA_A$ receptors with different subunit compositions—α1β2γ2, α2β2γ2, α3β2γ2 or α5β2γ2. Current decay time was fitted by the exponential equation $i(t) = \text{imax} * e^{(-t/\tau)}$, where imax is the maximal current of that cell, t is the time (s) and τ is the time constant of current decay and normalized to percent of effect to baseline control (perfusion of 0.1% DMSO) for each individual cell. These experiments were performed in the presence of GABA. Control is defined as the baseline applications of GABA before test item applications. The decay time was fit between the onset and offset of the GABA or GABA with test item application. **A** chlornordiazepam (CND), **B** mexazolam (MEX), **C** alprazolam (ALZ), **D** bromazepam (BRO), **E** zolpidem (ZLP). Closed circles represent α1β2γ2, open circles represent α2β2γ2, closed squares represent α3β2γ2 and open squares represent α5β2γ2. A one-way ANOVA followed by Dunnett's multiple comparison test was used to compare each concentration with baseline control (current decay time in the presence of 0.1% DMSO), $p < 0.05$ ($n = 3$–6); [a]statistically different from control for α1β2γ2; [b]statistically different from control for α2β2γ2, [c]statistically different from control for α3β2γ2; [d]statistically different from control for α5β2γ2

Table 3 Individual selectivity considering the effect of the different compounds

Different compounds tested	$GABA_A$ receptors with different subunit compositions			
	α1β2γ2	α2β2γ2	α3β2γ2	α5β2γ2
CND	–	+	+	+
MEX	+	–	–	–
ALP	+	–	+	–
BRO	+	–	+	+
ZLP	+	+	–	–

CND, MEX, ALZ, BRO and ZLP—on increasing peak current amplitude of $GABA_A$ receptors with different subunit compositions—α1β2γ2, α2β2γ2, α3β2γ2 or α5β2γ2

CND Chlornordiazepam, *MEX* mexazolam, *ALZ* alprazolam, *BRO* bromazepam and *ZLP* zolpidem. +Positive allosteric modulator (PAM); −No effect at the highest concentration tested

and the more complex and heterogeneous biologic environment of the synaptic cleft.

In conclusion, the effect of ALZ and BRO, but not CND, upon the current amplitude of α1 $GABA_A$-containing receptors, may explain why ALZ and BRO are more prone than CND to promote sedative adverse events, and, why they are endowed with more interference on psychomotor performance. In addition, the fact that CND targets $GABA_A$ receptors containing both α2 and α3 subunits—subunits that have both been linked to anxiolytic effects—may render MEX a more effective anxiolytic when compared to ALZ and BRO, which were devoid of effects upon $GABA_A$ receptors containing α2 subunits. Despite the non-clinical nature of this study, this data provides experimental support to the clinical findings already made available. Currently, in the clinical context of a lack of selective anxiolytic drugs, benzodiazepines are still relevant drugs worldwide for the treatment of anxiety. Therefore, besides clinical and pharmacokinetic data, i.e. half-life/action duration: longer duration for anxiety, short duration for insomnia, currently widely used in clinical practice; knowing the individual affinity of each benzodiazepine towards $GABA_A$ receptors containing different α subunits, "pharmacodynamic fingerprint", is also critical for a more rational, tailormade and effective treatment. These and other similar electrophysiological findings, together with all known pharmacokinetic information, will contribute to the knowledge of a distinct pharmacological "fingerprint" of each benzodiazepine.

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Author contributions HF, VB, MJB, MAV and PSS: conceptually designed the study, wrote the protocol and the first draft of the manuscript. SH and EB: conducted the laboratory experiments and wrote the study report. All authors contributed to and have approved the final manuscript.

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Data availability The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest Hélder Fernandes, Vânia Batalha, Maria João Bonifácio and Patrício Soares-da-Silva were employees of BIAL—Portela & C[a] S.A at the time of the study. Simon Hebeisen and Ellen Braksator were employees of B'SYS GmbH at the time of the study. B'SYS GmbH received a grant from BIAL—Portela & C[a], S.A.

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