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RESEARCH PAPER

Role of α1- and α2-GABA$_A$ receptors in mediating the respiratory changes associated with benzodiazepine sedation

S Masneuf, J Buetler, C Koester and F Crestani

Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland

Correspondence
Dr Florence Crestani, Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. E-mail: crestani@pharma.uzh.ch

Authors' present addresses: SM, Institute of Anatomy, University of Zurich, Winterthurerstrasse 190, 8057 Zurich; JB, Bremgartnerstrasse 56, 8003 Zurich, Switzerland.

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BACKGROUND AND PURPOSE

The molecular substrates underlying the respiratory changes associated with benzodiazepine sedation are unknown. We examined the effects of different doses of diazepam and alprazolam on resting breathing in wild-type (WT) mice and clarified the contribution of α1- and α2-GABA$_A$ receptors, which mediate the sedative and muscle relaxant action of diazepam, respectively, to these drug effects using point-mutated mice possessing either α1H101R- or α2H101R-GABA$_A$ receptors insensitive to benzodiazepine.

EXPERIMENTAL APPROACH

Room air breathing was monitored using whole-body plethysmography. Different groups of WT mice were injected i.p. with diazepam (1–100 mg·kg^{-1}), alprazolam (0.3, 1 or 3 mg·kg^{-1}) or vehicle. α1H101R and α2H101R mice received 1 or 10 mg·kg^{-1} diazepam or 0.3 or 3 mg·kg^{-1} alprazolam. Respiratory frequency, tidal volume, time of expiration and time of inspiration before and 20 min after drug injection were analysed.

KEY RESULTS

Diazepam (10 mg·kg^{-1}) decreased the time of expiration, thereby increasing the resting respiratory frequency, in WT and α2H101R mice, but not in α1H101R mice. The time of inspiration was shortened in WT and α1H101R mice, but not in α2H101R mice. Alprazolam (1–3 mg·kg^{-1}) stimulated the respiratory frequency by shortening expiration and inspiration duration in WT mice. This tachypnoeic effect was partially conserved in α1H101R mice while absent in α2H101R mice.

CONCLUSIONS AND IMPLICATIONS

These results identify a specific role for α1-GABA$_A$ receptors and α2-GABA$_A$ receptors in mediating the shortening by benzodiazepines of the expiratory and inspiratory phase of resting breathing respectively.

Abbreviation

WT, wild-type

Introduction

Classical benzodiazepines represent the major class of sedative–hypnotics used in the treatment of epilepsies, sleep and anxiety disorders. In addition to these therapeutic indications, they are used in various other medical conditions and practices that affect breathing, including sleep-disordered breathing, acute severe asthma, anaesthetic

premedication and mechanical ventilation. Paradoxically, the respiratory effects of benzodiazepines at therapeutic doses are still unclear, and the underlying $GABA_A$ receptor modulation is unknown. Human and animal studies have shown little to moderate alterations in the resting breathing pattern in either of the two directions, depression or stimulation, upon benzodiazepine treatment during wakefulness and sleep (Dalen *et al.*, 1969; Utting and Pleuvry, 1975; Prato and Knill, 1983; Longbottom and Pleuvry, 1984; Morel *et al.*, 1984; Schneider *et al.*, 1996; Carley *et al.*, 1998; Tulen and Man in't Veld, 1998; Bonnet *et al.*, 1990; Wettstein *et al.*, 1990; Pirnay *et al.*, 2008; Carraro *et al.*, 2009; Abdala *et al.*, 2010). Even ventilatory depression, defined as a reduction in tidal volume and/or an increase in partial pressure in carbon dioxide (CO_2), and tachypnoea have been reported in the same benzodiazepine-treated individuals during rest (Berggren *et al.*, 1987; Mora *et al.*, 1995; Cohn *et al.*, 1992). Occurrence of a change in the respiratory frequency or depth depends on the benzodiazepine used, its dosage and route of administration, and is often attributed to its other actions. Generally, the ventilatory depression associated with normal clinical sedative doses of benzodiazepines is rarely observed in the normal adult population (Litchfield, 1981). It resembles that occurring during sleep and is attributed to the drug-induced reduction in upper airway muscle tonus and coordination with the diaphragm (Bonora *et al.*, 1985; Leiter *et al.*, 1985; Molliex *et al.*, 1993). The tachypnoea associated with benzodiazepine sedation would result from a depressant drug action on the neural circuits controlling the respiratory cycle (Teppema and Baby, 2011). The few reports investigating the effects of benzodiazepines on the respiratory cycle in healthy human subjects breathing room air are inconsistent, describing either lengthening or shortening of inspiration duration or shortening of expiration duration (Clergue *et al.*, 1981; Morel *et al.*, 1984; Skatrud *et al.*, 1988). However, studies using decerebrated cat preparations have shown a concurrent shortening of the time of inspiration and of expiration consecutive to a reduction in the duration of burst activities in bulbar inspiratory and post-inspiratory neurons upon i.v. benzodiazepine treatment (Takeda *et al.*, 1989; Haji *et al.*, 1999).

Four distinct $GABA_A$ receptor subtypes consisting of two α(1, 2, 3 or 5), two β and one γ2 subunit mediate the actions of benzodiazepines. The α-subunit adjacent to the γ2-subunit provides drug sensitivity, with a histidine residue (α1-H101, α2-H101, α3-H126, α5-H105) being essential for high-affinity binding (Wieland *et al.*, 1992; Minier and Sigel, 2004). Replacement of the histidine by an arginine residue renders the corresponding point-mutated $GABA_A$ receptor subtype insensitive to benzodiazepine modulation (Kleingoor *et al.*, 1993; Benson *et al.*, 1998). Studies using knock-in mice possessing one single point-mutated α1H101R, α2H101R, α3H126R or α5H105R receptor subtype have uncovered the role of these four $GABA_A$ receptors in mediating the sedative (α1), anxiolytic (α2 and α3) and muscle relaxant (α2 and α5) actions of diazepam and related compounds (Atack, 2005; Möhler, 2006).

In this study we proposed to clarify the action of diazepam and alprazolam on resting breathing, as tested in non-restrained, quite awake wild-type (WT) mice, and further unravel the role of α1- and α2-$GABA_A$ receptor subtypes in mediating the respiratory drug effects using α1H101R and α2H101R mice. The high-potency benzodiazepine alprazolam is widely used in the clinic for its potent muscle relaxant and anxiolytic efficacy. Our results show that the two benzodiazepines, at sedative doses, stimulate the resting respiratory frequency by shortening either expiration and/or inspiration in WT mice. We further provide evidence that α1-$GABA_A$ receptors mediate the drug effect on the time of expiration, whereas α2-$GABA_A$ receptors are essential for drug modulation of the time of inspiration.

Methods

Animals

All animal care and experimental procedures were approved by the Cantonal Veterinary Office (animal welfare and use committee) in Zürich. A total of 85 129X1/SvJ mice, which served as WT controls, 41 α1H101R and 27 α2H101R mice were used. The mutants derived from 7 to 10 breeding pairs (>N20 backcrossing to 129X1/SvJ and homozygous on six to eight generations for each strain) maintained in the same environmental conditions (Rudolph *et al.*, 1999; Löw *et al.*, 2000). From weaning, the experimental animals were reared in collective cages with food and water *ad libitum* under standard 12 h day–night cycle conditions (light on at 7 h 00 min) in the testing room. They were tested at the age of 2 to 4 months.

Whole-cell patch-clamp recording

HEK 293 cells, cultured in plastic dishes (35 mm of diameter) in a humidified 95% O_2, 5% CO_2 atmosphere at 37°C for 3–4 days, were transiently transfected with rat cDNA αβγ combinations at the final concentrations of 0.4 (α and β subunits) and 1.2 (γ2 subunit) μg cDNA per dish using the Superfect Transfection Kit (Qiagen, Basel, Switzerland). Whole-cell recordings were performed 2 days after, as previously described (Benson *et al.*, 1998). The recording chamber was perfused with (in mM) 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 glucose and 10 HEPES (free acid), pH of 7.4. The patch-clamp pipettes contained (in mM) 120 CsCl, 1 $CaCl_2$, 11 EGTA, 4 MgATP and 10 HEPES (free acid), pH of 7.3. Dose–response curves were obtained by applying test solutions containing the approximate receptor subtype-specific GABA EC_{10} (3 μm for α1β2γ2 and α3β3γ2; 2 μm for α2β3γ2 and 1 μm for α5β3γ2) with increasing concentrations of diazepam (10^{-11}–10^{-6} M) or alprazolam (10^{-10}–10^{-6} M) using the SF-77B perfusion fast stepper device (Warner Instruments, Inc., Hamden, CT) according to Rabe *et al.* (2007). Each drug application lasted 4 s. Cell responses were recorded at a standard –60 mV cell holding-potential by a patch-clamp amplifier (Axopatch-1D, Axon Instruments, Foster City, CA, USA), low-pass filtered by an eight-pole Bessel filter at 1 kHz and digitized by a Digidata 1200 interface (Axon Instruments). The sample rate was of at least 1 kHz. Absolute GABA-evoked Cl^- currents recorded upon drug application were normalized to the responses evoked by GABA alone. Eight to 20 cells per concentration, per drug and per αβγ2 receptor combination were tested. Sigmoidal dose–response fitting with variable slope and automatic rejection of outliers was done with the Graph-

Pad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). The total number of observations analysed varied between 29 and 67.

Experimental design

Breathing was recorded using the constant flow-through whole-body plethysmography technique. Mice were placed in individual calibrated plethysmograph chambers (200 mL) (EMKA Technologies, Paris, France) supplied with a constant airflow (600 mL·min^{-1}) and maintained at a continuously monitored temperature of 28°C to 32°C between 9h00 min and 17 h00 min. The animals were left undisturbed in the plethysmograph chambers for approximately 1 h of adaptation. Once they displayed a resting breathing rate for several consecutive minutes, they were moved to standard individual cages for drug injection (3–4 min) and then replaced in their respective plethysmograph chamber for an additional 40 min. They were weighed before and at termination of the breathing session. WT mice were distributed in nine different groups according to the drug and the dose administered (vehicle; diazepam: 1, 3, 10, 30 and 100 mg·kg^{-1}; alprazolam: 0.3, 1 or 3 mg·kg^{-1}). α1H101R and α2H101R mice were distributed in four groups (diazepam: 1 or 10 mg·kg^{-1} and alprazolam: 0.3 or 3 mg·kg^{-1}). Each animal received only one drug injection.

O_2 consumption and CO_2 production

The amounts of O_2 and CO_2 in the plethysmograph chambers were sampled by a gas exchange system (Qubit Systems Inc., Kingston, Canada) for five consecutive minutes, two to three times before and after drug injection. The airflow rate was reduced to 300 mL·min^{-1} to optimize the measurement. One plethysmograph chamber contained no animal and served as a blank control.

Data analysis and statistics

Breathing parameters, including the respiratory frequency (breaths·min^{-1}), the time of expiration (ms), the time of inspiration (ms) and its integral representing tidal volume (μL), were processed with the EMKA datanalyst software version 2.3.3.5 (EMKA Technologies, Paris, France). Periods of quiet wakefulness were determined from direct observations of the animal's behaviours and postures and related to a range of variations in respiratory frequency, time of inspiration and time of expiration. Overt sleep episodes corresponded to the combination of a respiratory frequency <110 breaths·min^{-1} and a time of expiration >390 ms. Body movements and activity periods were associated with a time of inspiration <100 ms. We excluded these combinations and obtained time series from which two values per animal were calculated. The first, control pre-drug value corresponded to the average of the time series during the last 30 min preceding drug injection and the second post-drug value to the average of the time series starting 20 min after drug injection. O_2 consumption and CO_2 production mean rates (mL·min^{-1}) were obtained from data collected during the last 20 s of each 5 min sampling period and further used for the calculation of the individual's respiratory exchange ratio. About 35% to 40% of animals did not match the criteria for awake resting breathing and were excluded from data analysis.

Data were transformed in decimal logarithms. Tidal volumes and O_2 consumption and CO_2 production rates were normalized to the body weight. Repeated-measures one-way (dose) or two-way (dose by genotype) ANOVA with unweighted cells were used to analyse the dose–responses of diazepam and alprazolam in WT and mutant animals. Newman–Keuls's tests were used for *post hoc* mean comparisons. The pre-drug mean values ± SEM of the 167 animals tested were: respiratory frequency, 129 ± 1 breaths·min^{-1}; time of expiration, 338 ± 2 ms; time of inspiration, 142 ± 1 ms; tidal volume: 9.1 ± 0.2 μL·g^{-1} and body weight, 23.7 ± 0.1 g. The group sizes were: WT animals, Vehicle, n = 16; diazepam 1 mg·kg^{-1}, n = 15; 3 mg·kg^{-1}, n = 5; 10 mg·kg^{-1}, n = 17; 30 mg·kg^{-1}, n = 7; 100 mg·kg^{-1}, n = 7; alprazolam 0.3 mg·kg^{-1}, n = 10; 1 mg·kg^{-1}, n = 5 and 3 mg·kg^{-1}, n = 17; α1H101R mice, diazepam 1 mg·kg^{-1}, n = 13; 10 mg·kg^{-1}, n = 8; alprazolam 0.3 mg·kg^{-1}, n = 13; 3 mg·kg^{-1}, n = 7; α2H101R mice, diazepam 1 mg·kg^{-1}, n = 6; 10 mg·kg^{-1}, n = 6; alprazolam 0.3 mg·kg^{-1}, n = 8; 3 mg·kg^{-1}, n = 7. Results in figures are shown as pre-drug and post-drug mean values ± SEM. Percentages in text represent the percentage changes from pre-drug, resting values. Statistical significance was set as $P \leq 0.05$.

Drugs

Diazepam and alprazolam were prepared in a 0.3% Tween 80/saline solution (vehicle) and administered i.p. in a volume of 4 or 5 mL·kg^{-1} body weight.

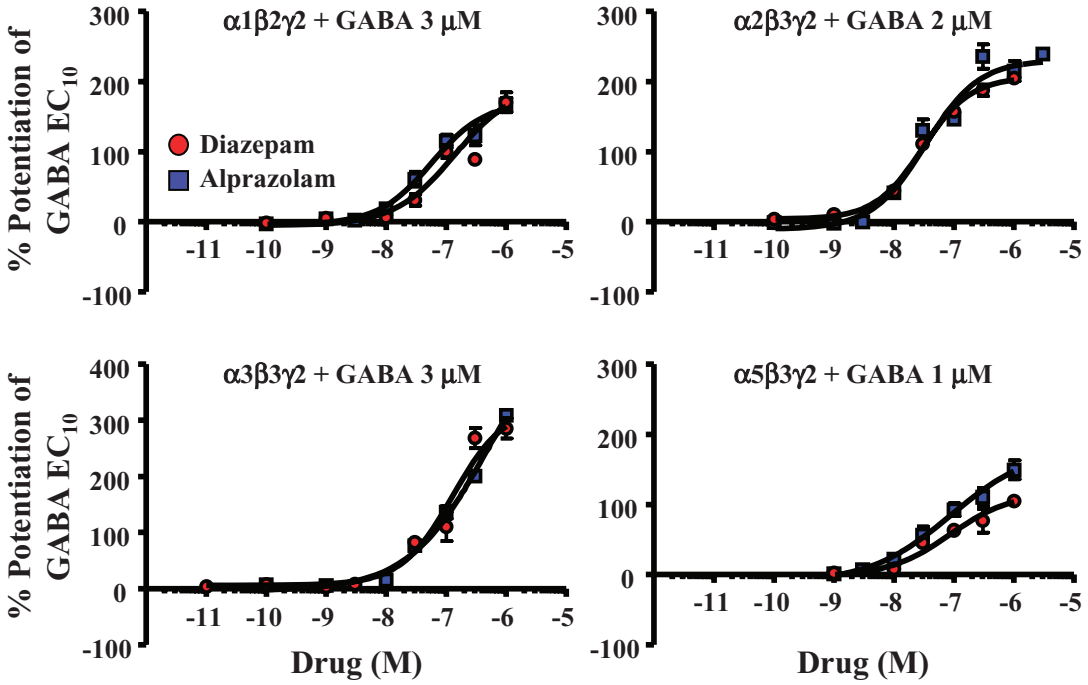
Results

Receptor profile of alprazolam

We compared the affinity and intrinsic efficacy of alprazolam with those of diazepam at α1β2γ2, α2β3γ2, α3β3γ2 and α5β3γ2 receptor combinations transiently expressed in HEK 293 cells. Data are shown in Figure 1. The potency and efficacy of alprazolam were in the range of that of diazepam at all four receptors. The highest maximal potentiation of GABA-evoked currents was obtained in α2β3γ2 and α3β3γ2 receptor combinations, with a Hill slope close to 1 for the two drugs.

Dose effects of diazepam on resting breathing in WT mice

We first analysed the respiratory effects of diazepam at doses (1–10 mg·kg^{-1}) inducing no to moderate motor sedation, as assessed on locomotor activity, in WT mice (Rudolph *et al.*, 1999). Upon diazepam treatment, mice displayed a rapid breathing pattern ($F_{(1,\ 49)}$ = 119, P < 0.001) with minimal changes in tidal volume (1 and 10 mg·kg^{-1}, 4%; 3 mg·kg^{-1}, –1%; Vehicle, –3%, $F_{(1,\ 49)}$ = 0.77, P = 0.38) (Figure 2A). The drug effect on the respiratory frequency reached significance from the dose of 3 mg·kg^{-1} (14%) and further increased with the dose of 10 mg·kg^{-1} (21%) ($F_{(3,\ 49)}$ = 18.45, P < 0.001) (Figure 3A). A shortening of the time of expiration was apparent at the three doses and augmented with the dose from 14% to 21% ($F_{(1,\ 49)}$ = 121, P < 0.001 and $F_{(3,\ 49)}$ = 11.04, P < 0.001) (Figure 3B). The changes in the time of inspiration were modest, varying from a slight lengthening (11%) in mice treated with 1 mg·kg^{-1} to a slight shortening (–7%) in



Receptor combinations	EC_{50} (nM)		Hill slope		Maximal Potentiation	
	Alprazolam	Diazepam	Alprazolam	Diazepam	Alprazolam	Diazepam
α1β2γ2	57 ± 13	137 ± 17	1.0 ± 0.2	0.9 ± 0.3	171 ± 12	196 ± 38
α2β3γ2	34 ± 13	30 ± 11	1.0 ± 0.2	1.0 ± 0.1	231 ± 14	207 ± 6
α3β3γ2	472 ± 19	129 ± 15	0.7 ± 0.1	1.0 ± 0.3	495 ± 105	326 ± 44
α5β3γ2	89 ± 22	85 ± 15	0.6 ± 0.4	0.9 ± 0.3	182 ± 52	117 ± 16

Figure 1

EC_{50}, Hill slope and maximal potentiation of GABA-evoked currents by alprazolam and diazepam at GABA EC_{10} for recombinant $GABA_A$ receptor subtypes expressed in transiently transfected HEK 293 cells.

those treated with 10 mg·kg^{-1} ($F_{(1, 49)}$ = 4.57, P = 0.04; $F_{(3, 49)}$ = 9.74, P < 0.001) (Figure 3C). No group differences were detected on any respiratory parameters before drug administration (Figure 3).

To test for a possible drug effect on metabolic respiration, we measured the rates of O_2 consumption and CO_2 production in some animals treated with vehicle, 1 or 10 mg·kg^{-1} diazepam (n = 5 mice per group), and found no alteration in the respiratory exchange ratio before and after drug administration, regardless of the dose (pre-drug/post-drug mean values ± SEM: Vehicle 0.67 ± 0.01/0.64 ± 0.03; 1 mg·kg^{-1} 0.66 ± 0.04/0.60 ± 0.02 and 10 mg·kg^{-1} 0.59 ± 0.02/0.58 ± 0.029; $F_{(2, 12)}$ = 0.78, P = 0.49).

Large sedative doses of benzodiazepines are used in minor surgery or as pre-anaesthetic medication. We therefore tested the effects of 30 and 100 mg·kg^{-1} diazepam on resting breathing in additional WT animals. Mice of the two groups displayed short expirations (–22% at both doses), short inspirations (–23% at 30 mg·kg^{-1} and –17% at 100 mg·kg^{-1}) and little changes in tidal volume (4% and 6%, respectively). Thus, in accordance with the difficulty of keeping the mice awake, these two drug treatments gave rise to a tachypnoea (29% and 25% above the resting respiratory frequency at 30 and 100 mg·kg^{-1}, respectively) associated with an increased mean inspiratory flow (Figures 2B and C).

Dose effects of diazepam on resting breathing in point-mutated mice

To identify the respective contribution of α1- and α2-$GABA_A$ receptor subtypes in mediating the tachypnoeic effect of diazepam, α1H101R and α2H101R mice were subjected to the same breathing and drug treatment (1 or 10 mg·kg^{-1}) protocols as WT animals. The analysis of the dose–responses to diazepam revealed significant genotype differences in the respiratory frequency ($F_{(2, 59)}$ = 3.75, P = 0.03) and the time of expiration ($F_{(2, 59)}$ = 8.73, P < 0.001). In α1H101R mice, the dose–response of the two parameters was impaired. α1H101R mice treated with the dose of 1 mg·kg^{-1} diazepam did not differ from their corresponding WT mice, showing short expirations, but conserved respiratory frequency (Figures 3A and

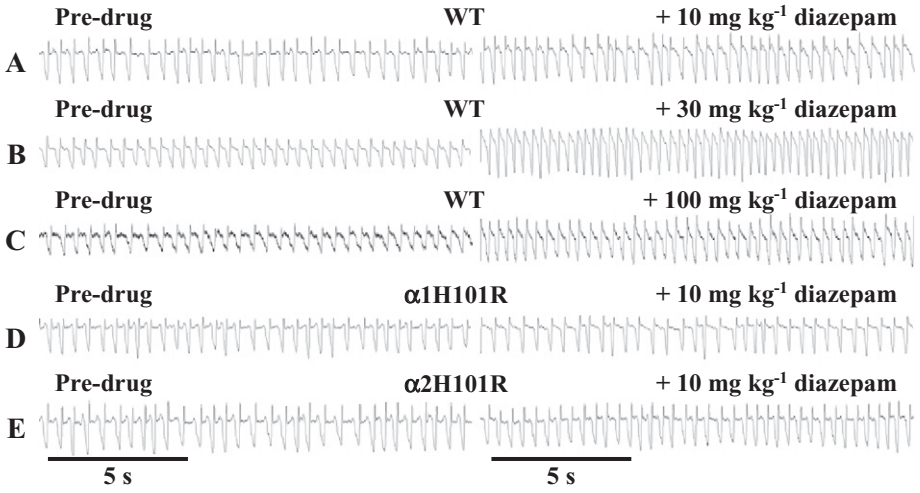


Figure 2

Plethysmographic recordings before (pre-drug) and upon treatment with different doses of diazepam in representative WT mice, α1H101R and α2H101R mice. The upward traces correspond to the expiratory phase and the downward traces to the inspiratory phases. The bar represents the time scale.

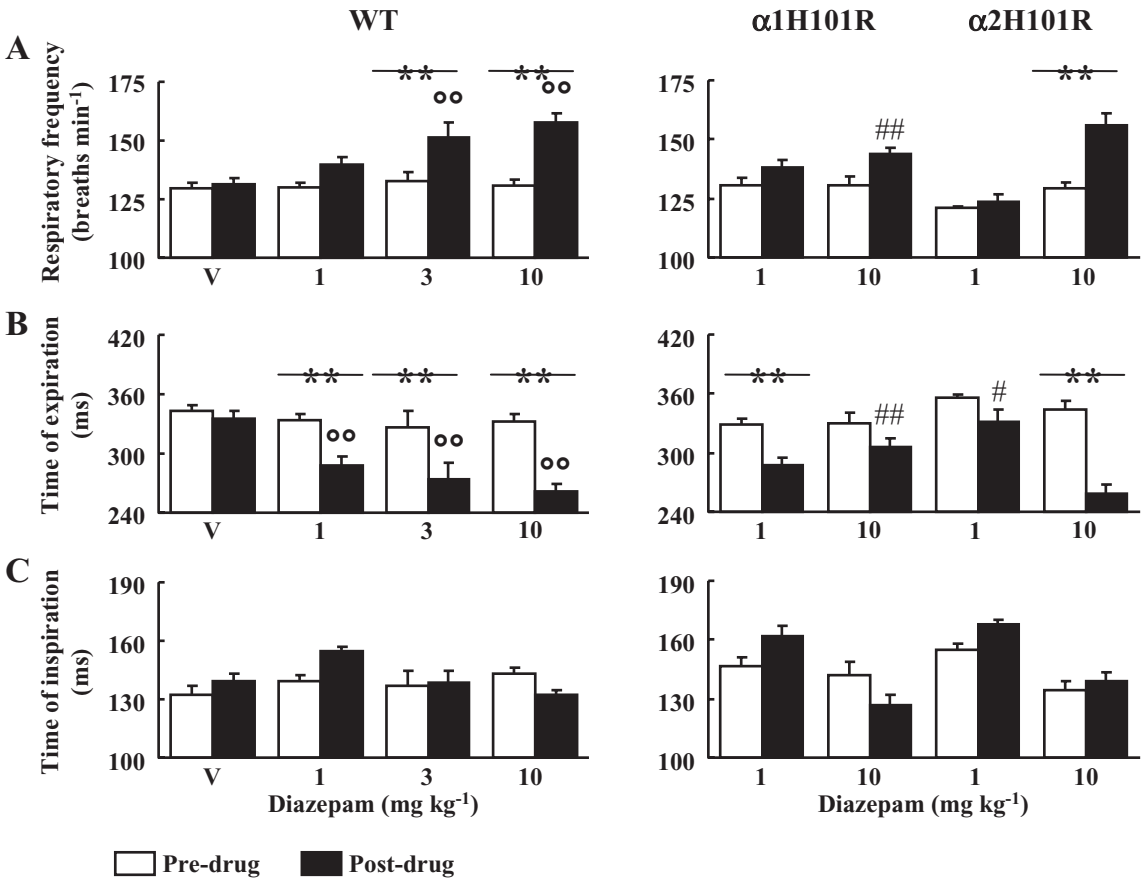


Figure 3

Respiratory frequency, time of expiration and time of inspiration before (pre-drug) and 20 min after i.p. injection of vehicle (V) or different doses of diazepam (post-drug) in WT mice, α1H101R and α2H101R mice. **$P < 0.01$ to pre-drug; °°$P < 0.01$ to V; #$P < 0.05$ and ##$P < 0.01$ to WT.

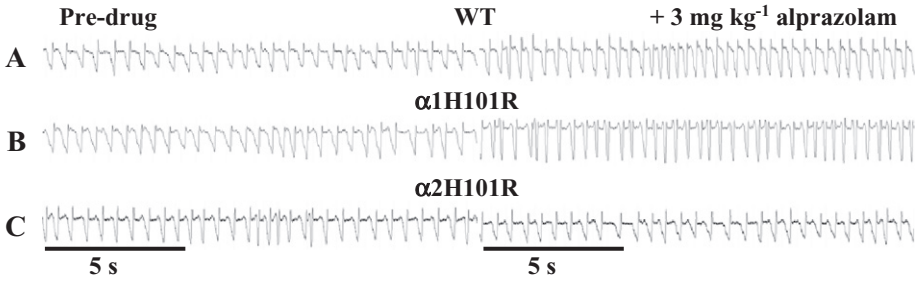


Figure 4

Plethysmographic recordings before (pre-drug) and upon 3 mg·kg^{-1} alprazolam treatment in representative WT mice, α1H101R and α2H101R mice. The upward traces correspond to the expiratory phase and the downward traces to the inspiratory phases. The bar represents the time scale.

B). α1H101R mice administered 10 mg·kg^{-1} diazepam displayed small changes in the respiratory pattern, with a respiratory frequency increased by 10% and a time of expiration shortened by –7% only (Figures 2D, 3A and B). Conversely, in α2H101R mice the dose of 1 mg·kg^{-1} diazepam was less effective in shortening the time of expiration (–7%), whereas the dose of 10 mg·kg^{-1} was associated with a tachypnoeic breathing pattern (21%) with short expirations (–25%) similar to that seen in WT animals (Figures 2E, 3A and B). As in WT mice, the time of inspiration was modestly affected after both drug treatments in the two mutants ($F_{(2,\ 59)} = 4.69$, $P < 0.02$) (Figure 3C).

Dose effects of alprazolam in WT and point-mutated mice

The range of doses of alprazolam was chosen to induce little to marked motor sedation, as previously reported in mice (Bourin *et al.*, 1992; Griebel *et al.*, 1996). Similar to diazepam, alprazolam at the doses of 1 and 3 mg·kg^{-1} induced a significant increase in the respiratory frequency (16% both doses) ($F_{(3,\ 44)} = 11.63$, $P < 0.001$), with minor alterations in tidal volume (0.3 mg·kg^{-1}, –9%; 1 mg·kg^{-1}, 1%; and 3 mg·kg^{-1}, 11%; $F_{(3,\ 44)} = 2.39$, $P = 0.08$) (Figures 4A and 5A). The tachypnoeic drug response resulted from concomitant shortening of the times of expiration (14% at 3 mg·kg^{-1}; $F_{(3,\ 44)} = 4.09$, $P = 0.01$) and inspiration (11% at 3 mg·kg^{-1}; $F_{(3,\ 44)} = 15.32$, $P < 0.001$), which reached significance only in mice administered 3 mg·kg^{-1} in comparison with vehicle controls (Figure 5B and C). We further examined the effects of 0.3 and 3 mg·kg^{-1} alprazolam on the resting breathing pattern in α1H101R and α2H101R animals. The analysis revealed significant dose by genotype interactions on the respiratory frequency ($F_{(2,\ 56)} = 5.29$, $P = 0.008$) and the time of inspiration ($F_{(2,\ 56)} = 7.35$, $P = 0.002$), but not on the time of expiration ($F_{(2,\ 56)} = 2.83$, $P = 0.07$). The dose of 0.3 mg·kg^{-1} alprazolam did not affect the resting breathing pattern in the two mutants (Figure 5). After treatment with 3 mg·kg^{-1} alprazolam, α1H101R mice showed short inspirations (–15%) but conserved time of expiration, giving rise to a modest increase in the respiratory frequency (9%) (Figures 4B and 5). Both drug effects were impaired in α2H101R mice such that their resting respiratory pattern remained unchanged after alprazolam treatment (Figures 4 and 5).

In summary, diazepam (1–10 mg·kg^{-1}), mainly by shortening the time of expiration, and alprazolam (0.3–3 mg·kg^{-1}), by shortening both the time of expiration and the time of inspiration, dose- dependently increased the resting respiratory frequency without altering tidal volume in WT mice. High sedative doses of diazepam (30–100 mg·kg^{-1}) were associated with an effortful tachypnoeic breathing pattern due to a marked shortening of the two phases of the respiratory cycle. In α1H101R mice, diazepam (10 mg·kg^{-1}) failed to induce tachypnoea and to reduce the time of expiration, though it was effective in shortening the time of inspiration. In α2H101R mice, the same drug treatment induced a tachypnoeic breathing pattern with shortened expirations similar to that seen in WT mice, in the absence of change in the duration of the inspiratory phase. The tachypnoeic effect of 3 mg·kg^{-1} alprazolam was partially retained in α1(H101R) mice, while absent in α2H101R mice.

Discussion and conclusions

Breathing is a rhythmic motor behaviour, which is essentially made up of inspirations and passive expirations driven by vagally mediated reflexes involving the diaphragm, intercostal and upper airway muscles during rest. $GABA_A$ receptor-mediated fast synaptic inhibition plays a major role in the modulation of these reflexive activities (Mizuta *et al.*, 2008; Sun *et al.*, 2008; Aleksandrova *et al.*, 2010) and of the central inspiratory off-switch mechanism, which controls the duration of inspiration and initiates expiration (Bonham, 1995; Haji *et al.*, 2000). Using an *in vivo* genetic strategy, we demonstrate for the first time a distinctive role for α1-$GABA_A$ receptors and α2-$GABA_A$ receptors in mediating the changes in the two phases of the respiratory cycle occurring upon benzodiazepine sedation. Specifically, drug activation of α1-$GABA_A$ receptors leads to expiration shortening, whereas α2-$GABA_A$ receptors participate in drug modulation of inspiratory duration.

We showed that diazepam and alprazolam in a range of doses inducing muscle relaxation and motor sedation (Bourin *et al.*, 1992; Griebel *et al.*, 1996; Rudolph *et al.*, 1999) consistently augment the resting respiratory frequency in WT mice. This result is in agreement with numerous studies describing a stimulant respiratory action of diazepam in rats (Carley *et al.*, 1998) and a tachypnoeic breathing pattern upon diazepam, midazolam, flurazepam, flunitrazepam or triazolam sedation in healthy human subjects during awake, rest or

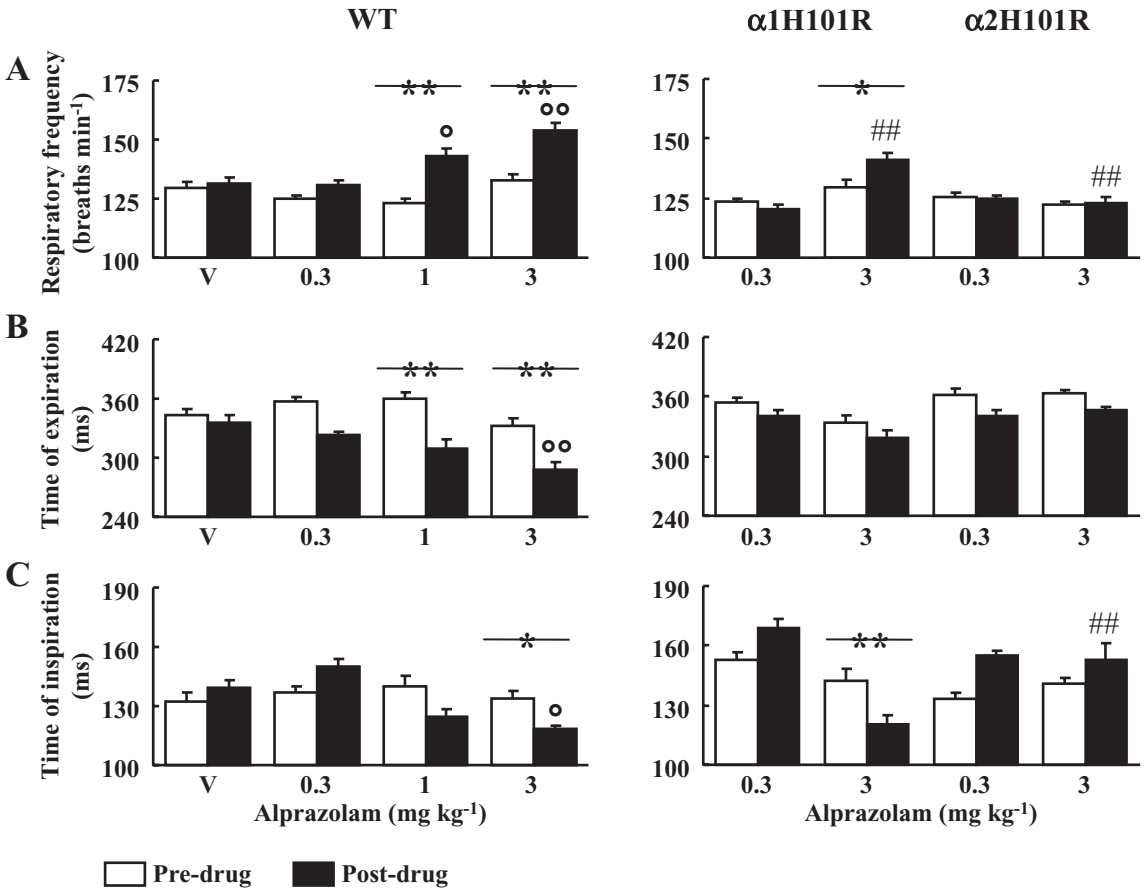


Figure 5

Respiratory frequency, time of expiration and time of inspiration before (pre-drug) and 20 min after i.p. injection of vehicle (V) or different doses of alprazolam (post-drug) in WT mice, α1H101R and α2H101R mice. *P < 0.05 and **P < 0.01 to pre-drug; °P < 0.05 and °°P < 0.01 to V; ##P < 0.01 to WT. The vehicle group shown in Figure 3 is represented for clarity.

sleep (Prato and Knill, 1983; Longbottom and Pleuvry, 1984; Morel *et al.*, 1984; Berggren *et al.*, 1987; Skatrud *et al.*, 1988; Cohn *et al.*, 1992; Mora *et al.*, 1995; Schneider *et al.*, 1996). However, it has also been reported that benzodiazepines have no effect on the respiratory frequency (Utting and Pleuvry, 1975; Wettstein *et al.*, 1990; Maillard *et al.*, 1992; Tulen and Man in't Veld, 1998; Carraro *et al.*, 2009). The dose and route of administration, the vehicle used for drug preparation and the post-drug time of testing are the many factors, which probably contribute to this discrepancy. Within the limits of our testing conditions, benzodiazepine sedation was not accompanied by a change in tidal volume and in the respiratory exchange ratio. This accords with the reported absence of alterations in tidal volume after administration of 1–2 mg·kg^{-1} midazolam in mice (Voituron and Hilaire, 2011) and in metabolic demands upon triazolam or flunitrazepam in humans (Skatrud *et al.*, 1988; Schneider *et al.*, 1996).

Next, we showed in WT mice that the tachypnoeic effect of diazepam (10 mg·kg^{-1}) is mainly due to a shortening of expiration whereas that of alprazolam (3 mg·kg^{-1}) is associated with similarly shortened expiration and inspiration. In agreement with our findings, a 35% shortening of expiration accompanied by a modest 4% shortening of inspiration upon midazolam sedation (Morel *et al.*, 1984) and a 20–25% shortening of both expiration and inspiration upon triazolam sedation (Skatrud *et al.*, 1988) have been reported in humans. These observations suggest some degree of specificity of the drug action regarding the two phases of resting breathing. However, we did observe a marked shortening of inspiration with larger sedative doses of diazepam (≥30 mg·kg^{-1}) in our animals. Likewise, in decerebrated cat preparations, i.v. diazepam or midazolam was shown to shorten inspiratory and expiratory duration to a similar extent via post-synaptic inhibition of bulbar inspiratory and post-inspiratory neural activities (Takeda *et al.*, 1989; Haji *et al.*, 1999).

The failure of α1H101R mice to display expiration shortening and tachypnoea upon diazepam treatment strongly implicates α1-GABA$_A$ receptors in mediating these two drug responses and corroborates their interdependence. This is in keeping with the conserved drug-induced shortening of inspiration in these mutants. The retained ability of α2H101R mice to show shortened expiration and tachypnoea upon diazepam sedation excludes the contribution of GABA$_A$ receptors other than α1-GABA$_A$ receptors in mediating these drug effects, given that α1H101R and α2H101R mice share benzodiazepine sensitive α3- and α5-GABA$_A$ receptors in common.

In line with our results, the α1-$GABA_A$ receptor antagonist β-CCT was shown to partially reverse the effects of midazolam on spontaneous breathing (Greenberg *et al.*, 1997). It is worth noting in this mouse study that midazolam sedation was associated with a bradypnoea and not a tachypnoea as reported in humans (Morel *et al.*, 1984). Based on our own observations, this discrepancy is probably attributable to the subject's actual behavioural state preceding drug administration. In the Greenberg *et al.* (1997) study, the animals exhibited a pre-drug mean respiratory frequency approximating 275 breaths·min^{-1}, which largely exceeds a mean resting respiratory frequency in this species (Nakamura *et al.*, 2003). In the Morel *et al.* study, the subjects were tested after a prolonged rest period. It is well known that α1-$GABA_A$ receptors represent the major molecular substrates underlying the benzodiazepine action on locomotion and motor coordination (Rudolph *et al.*, 1999; Crestani *et al.*, 2000, 2002; McKernan *et al.*, 2000). Our current study extends the significance of α1-$GABA_A$ receptors in the modulation of motor functions to that of the expiratory phase of resting breathing.

So far, our findings argue against a possible link between the tachypnoeic effect of diazepam and its muscle relaxant action as this latter drug effect is fully retained in α1H101R mice, as previously reported (Rudolph *et al.*, 1999). However, an increased inspiratory muscle relaxation may account for the shortening of the time of inspiration, as seen in WT mice upon alprazolam and more modestly upon diazepam sedation. A role for α2-$GABA_A$ receptors in mediating this respiratory drug response is supported by the failure of α2H101R mice to display shortened inspirations in response to the two benzodiazepines, whereas this drug effect is maintained in α1H101R mice, thereby excluding a possible involvement of α1- $GABA_A$ receptors. In keeping with the previously reported contribution of α2-$GABA_A$ receptors to the muscle relaxant action of benzodiazepines (Crestani *et al.*, 2001), these observations suggest a role for α2-$GABA_A$ receptor-mediated postsynaptic inhibition in the modulation of the Hering–Breuer inspiratory reflex, which initially involves activation of inspiratory neurons from the nucleus of the tractus solitarius by slowly adapting mechanoreceptors in response to lung inflation and/or of the central inspiratory off-switch mechanism, which terminates the inspiratory phase of the respiratory cycle.

In agreement with our findings, diazepam and other benzodiazepines with a similar non-selective receptor profile (Smith *et al.*, 2001) have shown some efficacy against apnoeas in animals and humans. Notably, in a rat model of central sleep apnoea it has been reported that diazepam effectively diminishes the occurrence of apnoeas during non-REM sleep (Carley *et al.*, 1998). Likewise, diazepam and midazolam were shown to reduce the amount of apnoeic episodes in a mouse model of Rett syndrome (Abdala *et al.*, 2010; Voituron and Hilaire, 2011). Interestingly, this mouse model is associated with a deficit of $GABA_A$ receptor α2 subunits but conserved α1-subunit expression in the brainstem (Medrihan *et al.*, 2008). In humans, triazolam and clonazepam were reported to be of benefit against idiopathic central sleep apnoea (Guilleminault *et al.*, 1988; Bonnet *et al.*, 1990). Conversely, alprazolam is a potent anxiolytic and muscle relaxant, which is often given in acute management of anxiety and panic attacks independently of the subject's respiratory nosology, which can vary from modest tachypnoeic breathing to hyperventilation or dyspnoea. A thorough analysis of its respiratory effects in susceptible patients is missing.

In conclusion, our study reveals an important role for α1-$GABA_A$ and α2-$GABA_A$ receptors in mediating benzodiazepine modulation of expiration and inspiration duration during rest. Also important is the finding that, depending on the preferential receptor profile of the benzodiazepine, one of these two $GABA_A$ receptor mechanisms may predominantly account for the resulting tachypnoeic breathing pattern. These results might help to refine the therapeutic uses of benzodiazepines and their limitations in clinical conditions associated with breathing alterations.

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Conflict of interest

None.

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