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SPECIAL REPORT

Mechanism of action of the hypnotic zolpidem *in vivo*

*,[1]Florence Crestani, [2]James R. Martin, [1]Hanns Möhler & [1]Uwe Rudolph

[1]Institute of Pharmacology and Toxicology, University of Zurich and Swiss Federal Institute of Technology (ETH) Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland and [2]Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

Zolpidem is a widely used hypnotic agent acting at the $GABA_A$ receptor benzodiazepine site. On recombinant receptors, zolpidem displays a high affinity to α1-$GABA_A$ receptors, an intermediate affinity to α_2- and α_3-$GABA_A$ receptors and fails to bind to α_5-$GABA_A$ receptors. However, it is not known which receptor subtype is essential for mediating the sedative-hypnotic action *in vivo*. Studying α1(H101R) mice, which possess zolpidem-insensitive α_1-$GABA_A$ receptors, we show that the sedative action of zolpidem is exclusively mediated by α_1-$GABA_A$ receptors. Similarly, the activity of zolpidem against pentylenetetrazole-induced tonic convulsions is also completely mediated by α_1-$GABA_A$ receptors. These results establish that the sedative-hypnotic and anticonvulsant activities of zolpidem are due to its action on α_1-$GABA_A$ receptors and not on α_2- or α_3-$GABA_A$ receptors.

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Abbreviations: GABA, γ-aminobutyric acid; PTZ, pentylenetetrazole; ANOVA, analysis of variance

Introduction The pharmacological profile of benzodiazepines and structurally unrelated ligands of the benzodiazepine site of $GABA_A$-receptors is dominated by anxiolytic, sedative, myorelaxant and anticonvulsant properties. In the search for ligands with a selective pharmacological profile two lines of research have been followed. (1) Partial agonists were thought to provide anxioselective ligands based on the assumption that the receptor reserve for anxiolytic activity would be higher than that for other pharmacological actions. While this concept produced promising results in animal experiments no partial agonist has been introduced into clinical practice (Haefely *et al*., 1990; Stephens *et al*., 1993). (2) The second approach was based on the recognition that $GABA_A$ receptors are divided into subtypes which differ in their subunit composition and regional distribution in the brain (for review, see Möhler, 2000; Möhler *et al*., 2000). The triazolopyridazine CL218872 was the first ligand which displayed a differential affinity for the benzodiazepine site, since it displaced [^{3}H]-diazepam with higher affinity from cerebellar than from hippocampal or cortical membranes (Lippa *et al*., 1979). The hypnotic zolpidem, an imidazopyridine, was the first subtype-selective ligand in clinical use. When tested on recombinant receptors, zolpidem displayed a high potency at α_1-$GABA_A$ receptors ($\alpha_1\beta_2\gamma_2$, $\alpha_1\beta_3\gamma_2$: $K_i = 20$ nM), medium potency at α_2- and α_3-$GABA_A$ receptors ($\alpha_2\beta_1\gamma_2$, $\alpha_3\beta_1\gamma_2$: $K_i = 400$ nM) but failed to interact with receptors containing the α_5 subunit ($\alpha_5\beta_3\gamma_2$, $\alpha_5\beta_2\gamma_2$: $K_i \geqslant 5000$ nM) (Langer *et al*., 1992; Pritchett & Seeburg, 1990). Thus, it was postulated that the pronounced sedative-hypnotic effect of zolpidem (Depoortere *et al*., 1986) was mediated by its preferential interaction with α_1-$GABA_A$ receptors. However, this view was in conflict with the characterization of the similarly α_1-selective imidazopyridine alpidem as a selective anxiolytic (Zivkovic *et al*., 1990; Morselli, 1993). The extraordinary strong GABA shift for zolpidem – an increase in the affinity of zolpidem by a factor of 3 in the presence of 100 μM GABA *in vitro* – was considered as alternative explanation for the preferential sedative-hypnotic effect of zolpidem (Arbilla *et al*., 1985; Depoortere *et al*., 1986). Thus, it has remained unclear whether the sedative-hypnotic effect of zolpidem *in vivo* is exclusively due to its interaction with α_1-$GABA_A$ receptors, or due to a combined interaction with α_1-, α_2- and α_3-$GABA_A$ receptors or whether it is mainly determined by its failure to modulate the neuronal populations expressing α_5-$GABA_A$ receptors. Therefore, in the present study, the subtype selectivity of the sedative effect of zolpidem was assessed *in vivo*. For this purpose, a mouse line was used in which the benzodiazepine binding site of α_1-$GABA_A$ receptors was rendered drug-insensitive by the introduction of a histidine to arginine point mutation in position 101 [α_1(H101R)] (Rudolph *et al*., 1999). In these animals, those pharmacological effects of zolpidem which are mediated *via* α_1-$GABA_A$ receptors are expected to be absent, while potential drug effects mediated *via* α_2- and α_3-$GABA_A$ receptors would remain apparent. For comparison, we also included the classical non subtype-selective benzodiazepine diazepam in our analysis (see also Rudolph *et al*., 1999).

Methods *Animals* Wild type and α_1(H101R) mice (third backcross of the 129/Ola chimeras to the 129/SvEv background) were generated as described in Rudolph *et al*. (1999). Male and female mice were raised in group-housed cages (8–10 mice per cage (either under normal 12 h day–night cycle conditions (light on at 06.00 h, motor activity test) or under reverse conditions (light on at 20.00 h, anticonvulsant test). The behavioural tests were performed between 08.00 and 15.00 h. Food and water were provided *ad libitum*. At the time of testing the body weight was about 18–22 g.

Behavioural procedures Motor activity was recorded for an hour in individual plexiglas chamber ($20 \times 40 \times 31$ cm) using a Digiscan Animal Activity Monitoring system (Omnitech Electronics Inc., Columbus, OH, U.S.A.) 5 min after oral administration of vehicle, diazepam (3–30 mg kg^{-1}) or zolpidem (10–60 mg kg^{-1}).

*Author for correspondence; E-mail: crestani@pharma.unizh.ch

The anticonvulsant activity of diazepam (3, 10 and 30 mg kg^{-1} p.o.) and zolpidem (3, 10 and 30 mg kg^{-1} i.p.) were tested against pentylenetetrazole (PTZ) (Depoortere *et al.*, 1986). This convulsant drug, when injected intraperitoneally at the dose of 120 mg kg^{-1} provokes myoclonic jerks associated with generalized tonic convulsions leading to death within a few seconds in all control mice. Diazepam and zolpidem were administered 30 min before PTZ. The number of mice developing the lethal tonic convulsion and/or myoclonic jerks within 10 min was noted.

Drugs Diazepam and zolpidem were suspended in a 0.3% Tween 80/saline solution. Pentylenetetrazole (PTZ, Sigma, Buchs, Switzerland) was dissolved in saline. All drugs were administered in a volume of 5 ml kg^{-1}.

Data analysis Continuous random variables were analysed using two-way ANOVAs followed by Fisher's pair-wise comparisons when appropriate. Chi-Square analysis and Fisher's exact tests were used for dichotomic variables (Conover, 1999).

Results *Influence of diazepam on motor activity in wild type and α_1(H101R) mice* Under vehicle treatment, wild type and α_1(H101R) mice displayed a similar level of motor activity. When increasing doses of diazepam were administered, the motor activity was decreased in a dose-dependent manner in wild type mice. Wild type mice treated with 10 and 30 mg kg^{-1} of diazepam were less active in comparison with their vehicle-treated controls ($P<0.01$) (Figure 1A). However, as reported previously (Rudolph *et al.*, 1999), the ability of diazepam to reduce motor activity was abolished in α_1(H101R) mice (Figure 1A). Up to the highest dose tested all α_1(H101R) mice showed the same amount of motor activity as the vehicle controls. ANOVA revealed a significant genotype-treatment interaction [$F(3, 120)=4.90$, $P<0.003$].

Effect of zolpidem on motor activity in wild type and α_1(H101R) mice In wild type mice, zolpidem depressed motor activity in a dose-dependent manner ($P<0.01$ versus vehicle) similar to diazepam (Figure 1B). However, in α_1(H101R) mice zolpidem up to 60 mg kg^{-1} did not decrease motor activity. ANOVA showed a significant genotype-treatment interaction [$F(3, 120)=3.27$, $P<0.02$]. A separate analysis of the dose-response curve of zolpidem in α_1(H101R) mice revealed no significant overall treatment effect on motor activity in α_1(H101R) mice [ANOVA, $F(3, 60)=1.91$, n.s.]. Again, no genotype difference was observed in response to vehicle injection.

Anticonvulsant activity of diazepam in the pentylenetetrazole test in wild type and α_1(H101R) mice Wild type mice were dose-dependently protected by diazepam from the lethal tonic convulsion provoked by PTZ [$\chi^2=21.79$, $P=0.001$] (Figure 2A). Similarly, the number of wild type animals showing myoclonic jerks was dose-dependently decreased following diazepam treatment [$\chi^2=31.11$, $P<0.001$] (Figure 3A). At 30 mg kg^{-1} diazepam, all wild type mice were fully protected from both tonic and myoclonic convulsions. However, in α_1(H101R) mice diazepam displayed a statistically significant activity against the lethal tonic convulsion only at the highest dose, 30 mg kg^{-1}, at which 55% of the mice were protected [$P<0.001$ compared to vehicle, Fisher's exact test; $\chi^2=7.53$, $P<0.056$] (Figure 2A). Furthermore, diazepam displayed a dose-dependent partial activity against myoclonic jerks in α_1(H101R) mice [$\chi^2=12.08$, $P<0.01$]. At the highest dose tested (30 mg kg^{-1}), 37% of the mutant mice were protected from myoclonic jerks [$P<0.05$ versus vehicle] (Figure 3A). Thus, the anticonvulsant activity of diazepam in the PTZ convulsion test is partially due to its action on α_1-$GABA_A$ receptors, in particular at low doses (10 mg kg^{-1}), but $GABA_A$ receptors other than α_1 (i.e. α_2-, α_3- and/or α_5-$GABA_A$ receptors) clearly contribute to the full protective effect seen at the highest dose (30 mg kg^{-1}) in wild type mice.

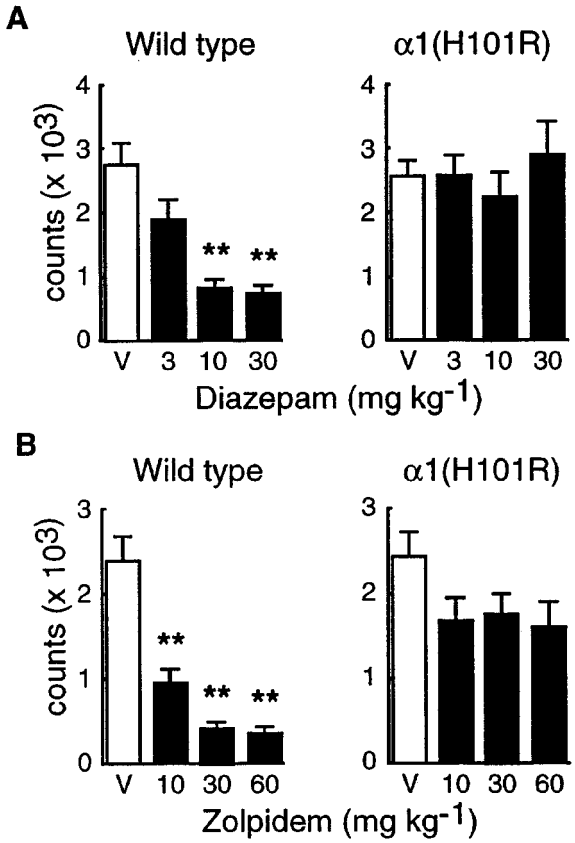

Figure 1 Effect of (A) diazepam (3–30 mg kg^{-1}) and (B) zolpidem (10–60 mg kg^{-1}) on motor activity in wild type and α_1(H101R) mice. Motor activity was recorded for 1 h after drug administration. Results are expressed as mean counts $\times 10^3 \pm$s.e.mean. $n=16$ mice per group. V, vehicle; $^{**}P<0.01$, Fisher's tests.

Effectiveness of zolpidem in the pentylenetetrazole test in wild type and α_1(H101R) mice In wild type mice zolpidem was protective against the lethal tonic convulsion in a dose-dependent manner [$\chi^2=29.45$, $P<0.001$] (Figure 2B). This effect was not observed in α_1(H101R) mice even at the highest dose tested (30 mg kg^{-1}) [$\chi^2=3.07$, n.s.]. This result suggests that the protective effect of zolpidem against the lethal tonic convulsion is mediated *via* α_1-$GABA_A$ receptors. In contrast, myoclonic jerks, which were assessed irrespective of whether they were followed by tonic convulsions or not, failed to be suppressed by zolpidem in the wild type mice even at the highest dose tested (30 mg kg^{-1}) [$\chi^2=6.31$, n.s.] (Figure 3B). This was the more surprising as α_1-$GABA_A$ receptors are occupied by zolpidem under these experimental conditions (Figure 1B). In α_1(H101R) mice, zolpidem was similarly ineffective in suppressing myoclonic jerks [$\chi^2=3.07$, n.s.].

Discussion Classical benzodiazepine hypnotics interact with comparable affinity with all drug-sensitive $GABA_A$-receptor subtypes (Kupfer & Reynolds, 1997; Benke *et al.*, 1996). In contrast, the imidazopyridine hypnotic zolpidem is a ligand with a preferential affinity for α_1-$GABA_A$ receptor subtype

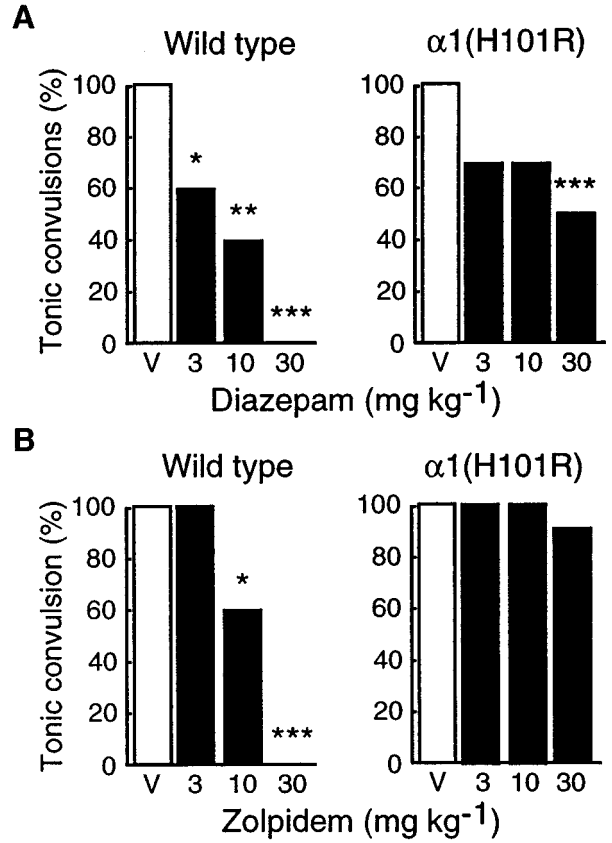

Figure 2 Effect of (A) diazepam (3–30 mg kg^{-1}) and (B) zolpidem (3–30 mg kg^{-1}) against the lethal tonic convulsion induced by pentylenetetrazole (120 mg kg^{-1}) in wild type and α_1(H101R) mice. Results are expressed as percentage of mice developing the tonic convulsion. $n=10-11$ per group. V, vehicle. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, Fisher's exact tests.

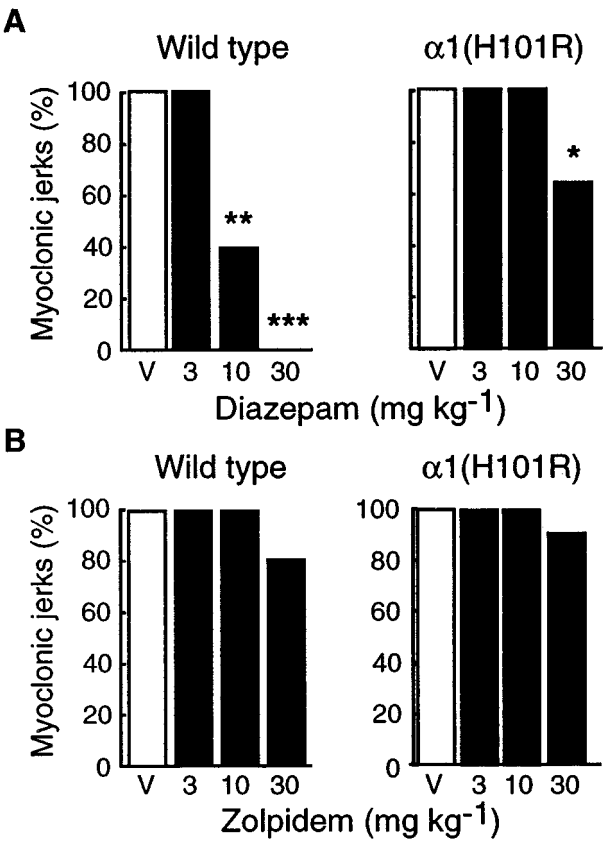

Figure 3 Effect of (A) diazepam (3–30 mg kg^{-1}) and (B) zolpidem (3–30 mg kg^{-1}) against the myoclonic jerks induced by pentylenetetrazole (120 mg kg^{-1}) in wild type and α_1(H101R) mice. Results are expressed as percentage of mice developing myoclonic jerks. $n=10-11$ per group. V, vehicle. $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, Fisher's exact tests.

as determined by radioligand binding studies *in vitro* (Arbilla *et al*., 1985; Depoortere *et al*., 1986; Bartholini, 1993). However, it had not been clarified whether its interactions with α_1-$GABA_A$ receptors is indeed the basis for its sedative-hypnotic activity. To assess the pharmacological relevance of the α_1-$GABA_A$-receptor subtype for the effectiveness of zolpidem *in vivo* an exquisite tool has become available through the recent development of a mouse line containing a knock-in point mutation in the benzodiazepine binding site. The α_1(H101R) point mutation renders the benzodiazepine binding site of α_1-$GABA_A$ receptors insensitive to classical benzodiazepines *in vivo* (Rudolph *et al*., 1999; McKernan *et al*., 2000). Similarly, binding of zolpidem to the point-mutated α_1-$GABA_A$ receptor is virtually abolished as determined after immunoprecipitation of α_1-$GABA_A$ receptors from α_1(H101R) mice. Zolpidem displayed a K_i value of 13 nM at wild type α_1-$GABA_A$ receptors and a K_i value of >10 μM at α_1(H101R) receptors (Rudolph *et al*., 1999). Mice with a point mutation (H101R) in the α_1 subunit are therefore suitable to test the contribution of α_1-$GABA_A$ receptors to the pharmacology of zolpidem *in vivo*.

The sedative effect of zolpidem was found to be entirely mediated by α_1-$GABA_A$ receptors at least up to a dose of 60 mg kg^{-1} as shown by the failure of zolpidem to significantly reduce motor activity in α_1(H101R) mice (Figure 1B). The result is in line with the finding that the sedative effect of diazepam is likewise mediated *via* α_1-$GABA_A$-receptors (Figure 1A) (Rudolph *et al*., 1999). Thus, the neuronal populations which express α_1-$GABA_A$ receptors mediate the sedative action of agonists acting at the benzodiazepine site irrespective of their chemical structure.

Apart from its sedative-hypnotic activity zolpidem had been characterized as a weak anticonvulsant active against the lethal PTZ-induced tonic convulsion (Depoortere *et al*., 1986). In the present study zolpidem was effective in reducing the PTZ-induced tonic convulsions in wild type mice with a potency comparable to that of diazepam. This anticonvulsant effect was abolished in α_1(H101R) mice, suggesting that the protection by zolpidem against tonic convulsions is likewise mediated *via* α_1-$GABA_A$ receptors (Figure 2B). However, even at the highest dose tested zolpidem failed to show anticonvulsant effectiveness against myoclonic seizures not only in α_1(H101R) mice but also in wild type mice (Figures 2B and 3B). This occurred despite the fact that α_1-$GABA_A$ receptors in wild type mice were occupied under the drug concentrations used. This failure by zolpidem to display activity against myoclonic jerks may be due to its reduced affinity to α_2- and α_3-$GABA_A$ receptors and/or its lack of interaction with α_5-$GABA_A$ receptors.

In summary, the present results clearly show that the sedative effect of zolpidem is mediated *via* α_1-$GABA_A$ receptors *in vivo*. This conclusion is of relevance for the development of future selective hypnotics acting at the benzodiazepine site.

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