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Rotarod studies in the rat of the $GABA_A$ receptor agonist gaboxadol: lack of ethanol potentiation and benzodiazepine cross-tolerance

Jeppe Voss[a], Connie Sanchez[a], Signe Michelsen[a], Bjarke Ebert[b,*]

[a] Department of Neuropharmacology, H. Lundbeck A/S, Ottiliavej 9, DK-2500 Valby, Denmark
[b] Department of Molecular Pharmacology, H. Lundbeck A/S, Ottiliavej 9, DK-2500 Valby, Denmark

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Abstract

All benzodiazepines and benzodiazepine site agonists impair motor performance dose-dependently and potentiate the effects of ethanol. In order to evaluate the risk of benzodiazepine and ethanol interaction with the direct acting $GABA_A$ receptor agonist 4,5,6,7-tetrahydroisoxazolo (5,4-*c*) pyridin-3-ol (gaboxadol), we studied impairment of motor coordination for combinations of gaboxadol, ethanol and a series of benzodiazepines (flunitrazepam, zolpidem and indiplon) in a rat rotarod model. All compounds produced a dose-dependent motor impairment and, in agreement with earlier data, a supra-additive effect of the benzodiazepine ligands and ethanol 1 g/kg was seen. In contrast, no significant potentiation of the effects of gaboxadol by ethanol was detected, and furthermore, no synergistic interaction between gaboxadol and any of the benzodiazepines was seen. A 30-day tolerance study was conducted with daily injections of gaboxadol (7.9 mg/kg) and zolpidem (1.25 mg/kg), respectively. A time-dependent tolerance developed to the motor impairment produced by both compounds. On day 31, cross-tolerance studies between zolpidem/gaboxadol and gaboxadol/zolpidem were conducted. No cross-tolerance was observed, indicating that the motor coordination effects observed with gaboxadol and zolpidem may arise from interaction with different receptor populations.

Keywords: Cross-tolerance; Ethanol; Flunitrazepam; $GABA_A$ receptor; Gaboxadol; THIP; Hypnotic; Rotarod; Zolpidem; Indiplon

1. Introduction

Benzodiazepines are widely used as hypnotics and in the treatment of anxiety and epilepsy. It is a group of drugs with low toxicity, but is also known to produce a number of side effects such as sedation, muscle relaxation, memory impairment, tolerance, dependence and risk of interaction with alcohol (Haefely, 1986; Woods et al., 1992). Efforts to overcome some of the unwanted effects have led to the development of a new class of benzodiazepine site agonists, including zolpidem, zaleplon and zopiclone (Goa and Heel, 1986; Langtry and Benfield, 1990; Sanger et al., 1996; Rosen et al., 1999; Drover et al., 2000).

These compounds are structurally different from the classical benzodiazepines; however, the pharmacological effect is mediated via the benzodiazepine site, located at the $GABA_A$ receptor complex. (Sanger et al., 1996; Rudolph et al., 1999; Crestani et al., 2000; McKernan et al., 2000).

Benzodiazepines and benzodiazepine site agonists exert their pharmacological effects via an allosteric modulation of the $GABA_A$/benzodiazepine receptor complex resulting in an enhanced GABAergic activity. $GABA_A$ receptors have a pentameric structure formed by co-assembly of different subunits. At present, at least 20 different subunits are known (α_{1-6}, β_{1-4}, γ_{1-3}, δ, ε, π, θ and ρ_{1-3}) (Barnard et al., 1998; McKernan and Whiting, 1996; Bonnert et al., 1999). The subunit composition of $GABA_A$ receptors varies throughout the brain. Most, but not all, $GABA_A$ receptors are formed by two α-subunits, two β-subunits and a γ-, ε- or δ-subunit (reviewed by Sieghart et al., 1999). The benzodiazepines actions are conferred to the interface between the α_1-, α_2-, α_3- or α_5-subunits and adjacent γ-subunit (Günther et al., 1995; Wingrove et al.,

* Corresponding author. Tel.: +45-36301311; fax: +45-36459356.
E-mail address: Bjeb@lundbeck.com (B. Ebert).

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1997). Two pharmacologically distinct benzodiazepine binding sites exist at the $GABA_A$ receptor, called BZ_1 and BZ_2 (Squires et al., 1979; Sieghart and Schuster, 1984) or ω_1 and ω_2 (Langer and Arbilla, 1988). Photoaffinity labelling experiments indicate that the BZ_1 site corresponds to $GABA_A$ receptors containing the α_1-subunit whereas the BZ_2 site corresponds to a population of receptors containing α_2-, α_3- or α_5-subunits. (Pritchett et al., 1989; Sieghart, 1995). The site to which GABA and other $GABA_A$ agonists binds is located at the interface between the α- and β-subunits (Pritchett et al., 1989; Pritchett and Seeburg, 1990). Reports have shown that the sedative/hypnotic effects may be mediated primarily by $GABA_A$ receptors containing the α_1-subunit (Sanger et al., 1994, 1996; Rudolph et al., 1999; McKernan et al., 2000), and the anxiolytic effects are believed to be primarily mediated by receptors containing α_2-, α_3- and α_5-subunits (McKernan et al., 2000).

In a series of sleep studies in animals and humans, Lancel and co-workers have characterised gaboxadol as a novel hypnotic with an effect distinctly different from that seen with benzodiazepines. Thus, in contrast to benzodiazepine site agonists, gaboxadol increases non-rapid eye movement (non-REM) sleep without suppressing REM sleep, improves the subjective quality of sleep and has no rebound effect after 5 days of treatment (Faulhaber et al., 1997; Lancel, 1997, 1999; Lancel and Faulhaber, 1996; Lancel et al., 1996; Lancel and Langelbartels, 2000). Evidence therefore suggests that gaboxadol may produce an activation of the $GABA_A$ receptor system, which is significantly different from allosteric $GABA_A$ receptor modulators like benzodiazepines.

In order to test if this unique mode of action of gaboxadol is translated into a novel interaction potential, the present study was carried out. In a rat model of motor performance, the rotarod model, the interactions between gaboxadol and chemically different types of benzodiazepine site agonists and the potentiation effects of ethanol on gaboxadol and benzodiazepine site agonists were characterised. Furthermore, in order to address the mechanisms underlying fading in tolerance to the motor impairment at high repeated dosing, long-term treatment with zolpidem and gaboxadol was carried out. These studies were designed so that a possible cross-desensitisation between gaboxadol and zolpidem could be investigated.

Compounds were chosen so that the classical benzodiazepine flunitrazepam, and the novel benzodiazepine ligands zolpidem and indiplon were included.

2. Materials and methods

2.1. Animals

Male Wistar rats (M&B, Denmark) weighing 150–200 g at the beginning of the studies were housed in macrolon cages, type III (425 × 266 × 180 mm) with two to four rats per cage (two per cage for animals weighing more than 200 g). Animals were acclimated for at least 4 days prior to experimentation and they were housed under a 12-h light–dark cycle (lights on 6 a.m.) and had standard pellet food and tap water available.

2.1.1. Acute studies

Rats were tested repeatedly allowing for at least a 72-h washout period between experiments.

Ethical permission for the studies were granted by the animal welfare committee, appointed by the Danish Ministry of Justice, and all animal procedures were carried out in compliance with the EC Directive 86/609/EEC and with the Danish law regulating experiments on animals.

2.1.2. Chronic studies

Animals included in chronic studies were treated as described above (Section 2.1.1) with the exception that access to food was restricted. This was done in order to ensure that the rats did not exceed a weight of 300 g, which is the limit for conducting studies on the rotarod. The animals were allowed a weight gain of 3.02 ± 0.09 g/day corresponding to 10–14 pellets/day/rat.

2.2. Drugs

Ethanol (Danisco, Denmark) and gaboxadol, HCl (Department of Medical Chemistry, H. Lundbeck, Denmark) were dissolved in 0.9% saline. Zolpidem and indiplon (Department of Medical Chemistry, H. Lundbeck) were dissolved in saline with a few drops of 0.1 N HCl added. Drug solution pH was then adjusted to pH>5 with 0.1 N NaOH. No adverse or toxic effects were observed at the injection site or behaviourally. Flunitrazepam (Department of Medical Chemistry, H. Lundbeck, Denmark, H. Lundbeck) was suspended in 10% methylcellulose. Control animals were injected with the corresponding vehicle.

Gaboxadol and zolpidem were injected subcutaneous (s.c.) while ethanol and flunitrazepam were injected intraperitoneal (i.p.) in an injection volume of 5 ml/kg.

Table 1
Motor performance deficits on the rotarod produced by gaboxadol and various $GABA_A$ receptor modulators

Treatment	ED_{50} mg/kg (95% confidence interval)
Gaboxadol, s.c.	6.4 (5.1–7.9)
Ethanol, i.p.	1600 (1300–2100)
Flunitrazepam, i.p.	4.63 (2.5–8.8)
Zolpidem, s.c.	0.56 (0.38–0.83)
Indiplon, s.c.	0.58 (0.31–0.87)

ED_{50} values were calculated by log-probit analysis. 95% confidence intervals are shown in brackets.

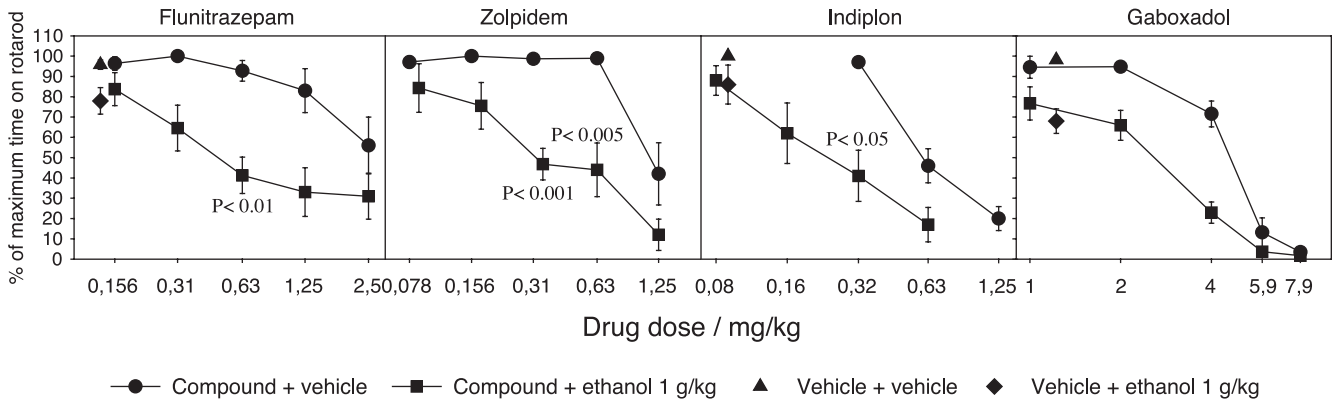

Fig. 1. Interaction studies between ethanol and gaboxadol and ethanol and a series of benzodiazepine site ligands measured in the rotarod model. Following administration of the test compounds, the acute effects on motor performance were measured. Results are shown as the percentage of the maximum possible running time on the rotarod with error bars indicating S.E.M. *P*-values refer to the effect of ethanol at the individual dose.

2.3. Rotarod motor coordination test

Motor performance was assessed by means of an automated rotarod (Rotamex 4/8, Columbus Instruments, Columbus, OH, and software and database developed by Ellegaard Systems, Faaborg, Denmark). The rats were trained and tested using a constant speed of 25 rpm (diameter of the rod: 75 mm). Before drug testing, the rats were trained daily for a 3-day period applying the following schedule: The first day, the rats were trained for 3 min with an unlimited number of trials on the rod followed by four trials of maximum 30 s with a 30-s inter-trial interval. On the second day, the rats were given five trials of maximum 30 s with a 30-s inter-trial interval. On the third day, the rats were given three trials of maximum 30 s with a 30-s inter-trial interval. On the third day, the accumulated time spent on the rod was measured and had to be at least 85 s to allow inclusion in the study. Drug testing was conducted at least 24 h after the final training trial. Pretreatment times were 15 min for ethanol, 30 min for gaboxadol, indiplon and zolpidem, and 45 min for flunitrazepam. During the tests, rats were given three trials of maximum 30 s with a 30-s inter-trial interval in the home cage. Latency to falling off the rod was recorded automatically for each animal by photocell detection.

Dose–response relationships were determined for ethanol (0.5–2.5 g/kg), gaboxadol (1.0–7.9 mg/kg), flunitrazepam (0.16–10 mg/kg), indiplon (0.08–0.63 mg/kg) and zolpidem (0.16–10 mg/kg).

Interaction studies were conducted between ethanol and gaboxadol or the benzodiazepine site ligands, and between gaboxadol and benzodiazepine site ligands.

The effects of repeated administration of gaboxadol (vehicle and 7.9 mg/kg) and zolpidem (vehicle and 1.25 mg/kg) were studied on the rotarod every 3–4 days for 30 days. Development of cross-tolerance was subsequently studied on day 31 in these groups of animals. Gaboxadol-treated rats were injected with 2.5 mg/kg zolpidem, whereas zolpidem-treated rats were injected with 7.9 mg/kg gaboxadol. When given acutely, these doses induced close to 100% impairment of the performance at the rotarod.

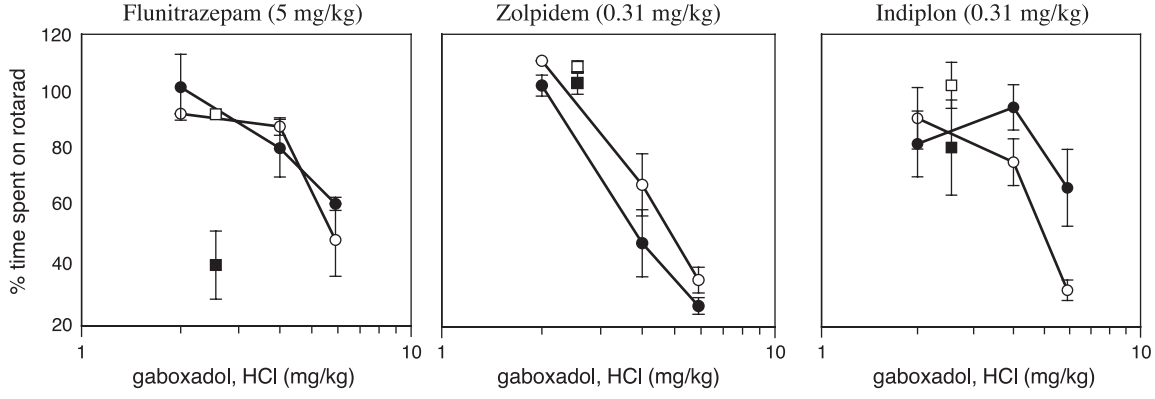

Fig. 2. Interaction studies between gaboxadol and a series of benzodiazepine site agonists measured in the rotarod model. Following administration of the test compounds, the acute effects on motor performance were measured. ○: gaboxadol; ●: gaboxadol plus benzodiazepine site agonist; □: vehicle; ■: benzodiazepine site agonist. For illustrative purposes, data for flunitrazepam have been corrected for the sedative effect of flunitrazepam, which is a 58% reduction. Results are shown as the percentage of the maximum possible running time on the rotarod with error bars indicating S.E.M.

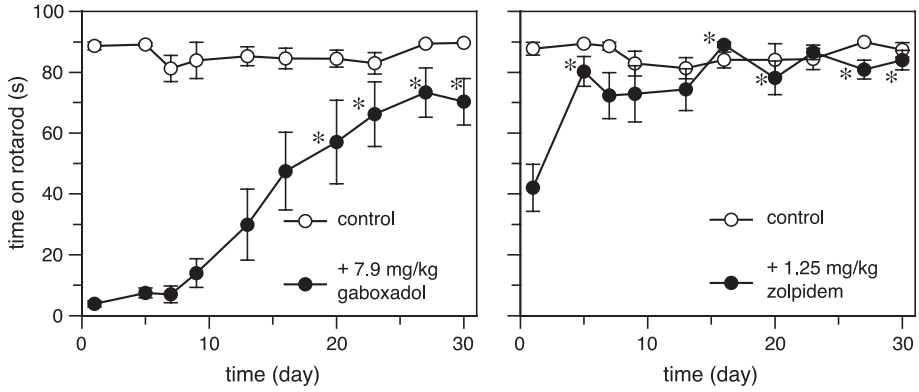

Fig. 3. Effect of repeated administration of gaboxadol or zolpidem on motor performance in the rotarod model. Single doses of gaboxadol (left) or zolpidem (right) were administrated each day throughout the 30-day period. ○: vehicle; ●: gaboxadol 7.9 mg/kg (left) or zolpidem 1.25 mg/kg (right). Results are shown as the running time on the rotarod with error bars indicating S.E.M. *Significantly different from starting value ($P<0.05$).

2.4. Statistics

ED_{50} values were calculated by means of log-probit analysis for the dose–response studies. The data from the dose response studies were further analysed by means of one-way analysis of variance (ANOVA) on ranked data since most data failed to be normally distributed or show homogeneity of variances. Where relevant, post hoc comparison was performed using Dunn's test.

Data from acute interaction studies were analysed by a two-way ANOVA.

The chronic data were evaluated using repeated measures one-way ANOVA, followed by pairwise comparisons of means using Dunn's method using the response on day 1 as control group. Missing values were replaced by group mean. Cross-tolerance studies were evaluated using a one-way ANOVA on ranks. A level of $P<0.05$ was considered statistically significant.

3. Results

3.1. Dose–response relationship

Gaboxadol, flunitrazepam, zolpidem and indiplon all produced a dose-dependent reduction in time spent on the rotarod. ED_{50} values are shown in Table 1.

3.2. Interaction studies

Flunitrazepam 0.63 mg/kg ($P<0.01$), zolpidem 0.31 mg/kg ($P<0.001$) and 0.63 mg/kg ($P<0.005$), and indiplon 0.31 mg/kg ($P<0.05$) all produced a significant facilitation of ethanol-induced motor deficits (1.0 g/kg, i.p.) (Fig. 1). Gaboxadol produced an additive rather than a potentiating effect of the ethanol-induced motor deficits (1.0 g/kg, i.p.) (Fig. 1) and no significant overall effects were observed between dose–response curves for gaboxadol and gabox-

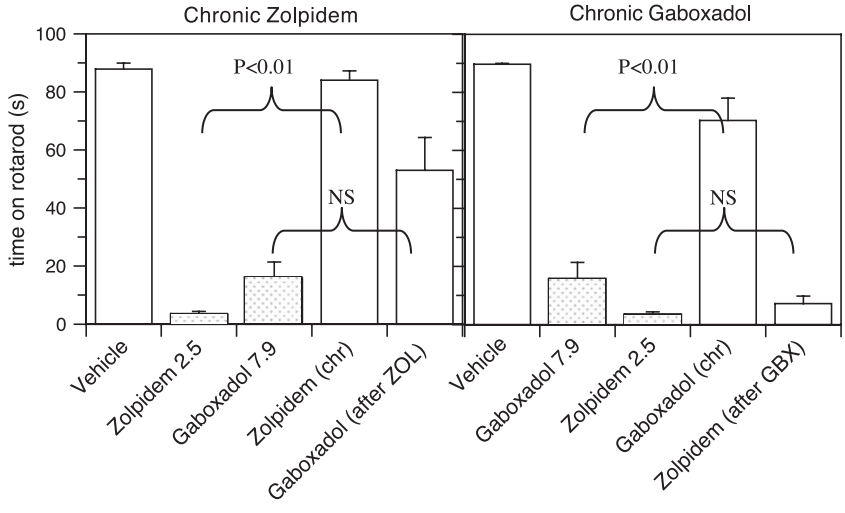

Fig. 4. Evaluation of cross-tolerance in rats previously treated with gaboxadol or zolpidem for 30 days. Rats treated with gaboxadol 7.9 mg/kg/day (left) or zolpidem 2.5 mg/kg/day were challenged with zolpidem or gaboxadol. In the graphs are included the effects of acute doses (dotted bar) of zolpidem and gaboxadol in groups of animals handled in parallel with the chronically treated animals. Results are shown as the percentage of the maximum possible running time on the rotarod with error bars indicating S.E.M.

adol plus ethanol (1.0 g/kg i.p.). Furthermore, gaboxadol did not significantly potentiate motor deficits produced by flunitrazepam, zolpidem and indiplon (Fig. 2).

3.3. Tolerance studies

As illustrated in Fig. 3, tolerance to the motor-impairment effects induced by 7.9 mg/kg gaboxadol and 1.25 mg/kg zolpidem developed during a 30-day treatment period. The speed by which tolerance developed was faster for zolpidem than for gaboxadol (Fig. 3).

3.4. Cross-tolerance studies

Following 31 days of treatment and the induction of tolerance to gaboxadol and zolpidem, cross-tolerance studies were conducted in the two treatment groups. No cross-tolerance between gaboxadol and zolpidem was observed, since the effect of a single dose of gaboxadol in rats tolerant to zolpidem did not differ significantly from the acute effect of the same dose in zolpidem- and gaboxadol-naive rats (Fig. 4) ($H(3)=5.342$, $P=0.148$; ANOVA on ranks (NS)). Similarly, the effect of zolpidem in gaboxadol-tolerant rats was not significantly different from that observed in gaboxadol- and zolpidem-naive rats (Fig. 4) ($H(3)=1.279$, $P=0.734$; ANOVA on ranks (NS))

4. Discussion

Gaboxadol has been used as a pharmacological tool within the $GABA_A$ receptor field for more than 20 years. From the original notion that gaboxadol was an agonist at the $GABA_A$ receptor, a much more complex pattern has emerged over the last 10 years. In vitro electrophysiological studies in *Xenopus* oocytes and cell lines have shown that the activity of gaboxadol is highly dependent on the receptor subunit composition. Thus, at the primarily synaptically located $\alpha1\beta3\gamma2$-containing receptors gaboxadol is a weak partial agonist with a maximum response of 70% (Ebert et al., 1997), relative to GABA and at the primarily extrasynaptically located $\alpha4\beta3\delta$ containing receptors gaboxadol is a high potent agonist with a maximum response of 200% relative to GABA (Brown et al., 2002; Adkins et al., 2001). In a more complex system like the rat cortical wedge preparation (Ebert et al., 2002; Storustovu and Ebert, 2003), where modulation of spontaneous activity has been characterised, gaboxadol acted as a highly potent agonist and, quite interestingly, did not show any synergistic interaction with the benzodiazepines. The interpretation of these findings was that, although gaboxadol as an agonist is able to activate all types of $GABA_A$ receptors, interaction with the highly abundant, highly gaboxadol sensitive and benzodiazepine insensitive extrasynaptic $\alpha4\beta3\delta$ containing receptors would determine the functional output (here: suppression of spontaneous activity) and thus produce an effect which is insensitive to modulation by benzodiazepines. However, qualitatively similar results and conclusions have been made with subtype selective benzodiazepines, so although in vitro data suggested receptor selectivity compared to the classical benzodiazepines, animal and human studies clearly demonstrated that this did not transform into any functional superiority (Landolt and Gillin, 2000). Therefore, in order to further characterise the interaction of gaboxadol with $GABA_A$ receptors in the whole animal, and determine if gaboxadol and benzodiazepines in rats do interact synergistically, the current study was conducted.

The classical benzodiazepine site agonist flunitrazepam, as well as the two novel benzodiazepine site agonists zolpidem and indiplon, produced a supra-additive enhancement of ethanol-induced motor coordination deficits (Fig. 1). These observations are in accordance with existing data in the literature, where the potentiating effect has been observed in humans (Chan, 1984; Woods et al., 1992; Roehrs et al., 2001) and in animals (Chan, 1984; Vanover et al., 1999). Ethanol is described to potentiate the effects of the classical benzodiazepines such as diazepam, triazolam and flunitrazepam to a greater extend than novel benzodiazepine site ligands such as zaleplon and zopiclone. (Seppälä et al., 1983; Kuitunen et al., 1990; Roehrs et al., 2001). However, in the present study, no difference in the degree of potentiation by the different benzodiazepine site ligands is seen. Our data therefore clearly suggest that under our test conditions, there is no difference in the interaction between ethanol and the different classes of benzodiazepine site agonists.

In contrast to these findings, gaboxadol was in the present study found to be devoid of any significant ethanol or benzodiazepine potentiating properties. Even at concentrations of gaboxadol where significant effects were observed, only additive effects of ethanol and the benzodiazepines were seen (Fig. 1).

Ethanol is believed to exert its effects on the $GABA_A$ receptor complex through allosteric modulation of the $GABA_A$ receptor (Davies et al., 1996, 1999) though the exact mechanism of action is unknown. Since both benzodiazepines and ethanol acts through allosteric modulation of the $GABA_A$ receptor, it is possible that concomitant administration results in further enhancement of GABA receptor gating resulting in a supra-additive response. An alternative explanation could be that ethanol enhances the penetration of the benzodiazepines into the brain thereby producing an apparent rightward shift in the dose–response curve. In favour of this hypothesis are earlier studies where co administration of ethanol and most benzodiazepines significantly increased the AUC of the benzodiazepine measured in peripheral blood (MacLeod et al., 1977; Sellers et al., 1980). However, others have shown that the concentration in brain of diazepam and chlordiazepoxide is only marginally affected by ethanol (Guthrie et al., 1987; Moriya and Ishizu, 1992). Furthermore, data for zolpidem have shown

that an increase in zolpidem binding is seen in the presence of ethanol (Devaud and Morrow, 1994; Devaud et al., 1995); however, in these studies, no brain levels are measured. Rastogi et al. (1986) suggest that the interaction takes place only at the receptor level, and in line with this are data from Klotz (1988) who reports that the concentration of flumazenil in the CNS is not affected by ethanol. In our opinion, data are therefore in favour of a pharmacological interaction at the receptor level and not a pharmacokinetic interaction leading to an increased level of benzodiazepine in the CNS. The increased binding of zolpidem in the CNS, reported by Devaud and co-workers, therefore most likely is a consequence of a conformational change in the $GABA_A$ receptor complex leading to an increased affinity for benzodiazepines without affecting the number of receptors. This type of mechanism has been reported by, e.g. Wafford et al. (1992) or Ticku et al. (1983). In contrast, gaboxadol as a direct acting $GABA_A$ receptor agonist (e.g. Ebert et al., 1994) does not work through allosteric coupling and thus possesses a mechanism of action different from that of benzodiazepines and ethanol. Although previous studies (Ebert et al., 1997) have shown that gaboxadol binds with equal affinity to different $GABA_A$ receptor subunit combinations and therefore the potential for a nonselective action is present, other studies (Ebert et al., 1994, 1997, 2002; Brown et al., 2002) have demonstrated that the functional consequences of GABA receptor binding are highly dependent on the actual subunit composition. Part of the explanation for differences between benzodiazepine site ligands and gaboxadol may therefore be ascribed to the differences in site of action at the receptor level.

In order to further test the hypothesis that the pharmacological effects of gaboxadol and zolpidem are mediated via different subpopulations of $GABA_A$ receptors, we therefore carried out a tolerance study in which very high doses of gaboxadol (7.9 mg/kg) and zolpidem (1.25 mg/kg) were used. If the mechanism underlying tolerance for the two compounds indeed were different, the prediction would be that no cross-tolerance would be present. As illustrated in Fig. 3, both compounds were able to induce tolerance. The sedative effects of zolpidem faded rapidly, whereas the effects of gaboxadol faded gradually over the 30 days. The mechanism underlying this induction of tolerance is still enigmatic, but has been ascribed to down-regulation of receptor number (Miller et al., 1988; Fahey et al., 2001) or reduced functional coupling (Tietz et al., 1989). In case of zolpidem, it could be hypothesised that the tolerance would be a consequence of enzyme induction and thus an altered overall bioavailability. However, 28 days of treatment with zolpidem in rats did not show any altered peak concentration in the brain or AUC (Trenque et al., 1994), ruling out a pharmacokinetic basis for the induction of tolerance. Gaboxadol is mainly excreted via the kidney (Schultz et al., 1981), so it is unlikely that changes in liver enzymes would affect the bioavailability. However, no studies have yet been reported with the aim of characterising induction of enzymes, so it cannot fully be ruled out that pharmacokinetic parameters, in part, are underlying the induction of tolerance.

As illustrated in Fig. 4, no cross-tolerance between gaboxadol and zolpidem was observed. Although not statistically significant, a trend towards a reduced acute effect of gaboxadol in animals chronically treated with zolpidem was present. It could be argued that complete cross-tolerance between gaboxadol and zolpidem in a complex model as the rotarod is very hard to demonstrate. However, Ramsey et al. (1991) using a similar paradigm were able to demonstrate cross-desensitisation between different classes of benzodiazepines. Furthermore, it can be argued that although gaboxadol under normal circumstances may exert its main action via extrasynaptic receptors, the very high doses used in the present experiment may activate synaptic receptors as well, wherefore some degree of cross-desensitisation might be expected. Despite of these caveats, when it still is possible to obtain a statistical significant lack of cross-tolerance, this clearly indicates that gaboxadol and zolpidem may mediate their effects via different transduction pathways, probably reflecting different receptor populations. Elegant studies by Rudolph et al. (1999) and McKernan et al. (2000) have shown that the sedative effects of benzodiazepines are mediated primarily via α1-containing $GABA_A$ receptors, which are supposed to be synaptically located. In light of our previous findings in the rat cortical wedge preparation (Ebert et al., 2002; Storustovu and Ebert, 2003), where a strong indication of extrasynaptic activity was the main responsible parameter for the activity of $GABA_A$ receptor agonists, it is very tempting to speculate that the two different $GABA_A$ receptor populations responsible for the action of zolpidem and gaboxadol are synaptic and extrasynaptic receptors, respectively. However, it still needs to be established if the receptor populations mediating impairment of motor coordination are identical to those mediating the sedative effects. Before these questions can be addressed, it still is a hypothesis that gaboxadol and zolpidem in the rotarod are interacting with distinctly different receptor populations.

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