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Gaboxadol — a different hypnotic profile with no tolerance to sleep EEG and sedative effects after repeated daily dosing

Bjarke Ebert [a,*], Neil J. Anderson [a], Thomas I. Cremers [c], Stine Rasmussen [d], Vanessa Vogel [a], Jeanne M. Fahey [d], Connie Sánchez [b]

[a] H. Lundbeck A/S, Ottiliavej 9, DK-2500 Valby, Denmark
[b] Lundbeck Research US, Paramaus, NJ, USA
[c] Brains-on-Line, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands
[d] Department of Pharmacology & Experimental Therapeutics Tufts University School of Medicine, Boston MA, USA

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Abstract

Gaboxadol, a selective extra synaptic $GABA_A$ receptor agonist, has been in clinical development for the treatment of insomnia. Development of tolerance to therapeutic effects (e.g. hypnotic and anticonvulsant and sedative) and withdrawal symptoms (e.g. REM sleep rebound and reduced seizure threshold) upon treatment discontinuation is reported for $GABA_A$ receptor allosteric modulators acting via the benzodiazepine binding site, e.g. zolpidem and indiplon. We conducted a head to head comparison in rats of the hypnotic (sleep EEG after 21 daily doses and 24 and 48 h after the last dose) and seizure threshold modifying (bicuculline assay 24 h after 28 daily doses) effects of gaboxadol and benzodiazepine ligands. Furthermore, we investigated in further details a previously reported apparent rapid development of tolerance to gaboxadol's effects in a rat rotarod motor coordination assay and related this effect to CNS exposure levels and *in vitro* potency at extra synaptic $GABA_A$ receptors.

Sleep EEG studies demonstrated lack of tolerance and withdrawal effects after 28 daily doses with gaboxadol, whereas zolpidem produced both tolerance and withdrawal effects under a similar dosing regimen. Daily dosing with gaboxadol, zolpidem or indiplon for 28 days and acute discontinuation of treatment left the threshold to bicuculline-induced seizures unchanged. The rapidly attenuated effect of repeated gaboxadol dosing was confirmed in the rotarod model. However, re-challenge of gaboxadol insensitive animals with gaboxadol produced a maximum response, ruling out that receptor desensitisation accounts for these effects. By comparing CNS exposure at rotarod responses and concentration response relation at cloned $GABA_A$ receptors expressed in *Xenopus* oocytes it appears that the decline in response in the rotarod model coincides with the steep part of the concentration response curve for gaboxadol at extra synaptic $GABA_A$ receptors.

In conclusion, rat sleep EEG repeated dose studies of gaboxadol confirm a hypnotic-like profile and no withdrawal effects, whereas tolerance and withdrawal effects were shown with zolpidem. Withdrawal from gaboxadol, zolpidem and indiplon did not affect the seizure threshold to bicuculline. Gaboxadol's apparent rapid development of tolerance in the rotarod assay appears to be kinetically determined.

Keywords: GABA; Extra synaptic receptor; Gaboxadol; In vivo; In vitro; Tolerance; Discontinuation

1. Introduction

Even though hypnotics modulating $GABA_A$ receptors have been in clinical use for more than four decades, novel $GABA_A$ receptor active hypnotics are still in development. Most recently the $GABA_A$ receptor agonist gaboxadol was in clinical development, but discontinued very late in the development program. Publicly available clinical data (Walsh et al., 2007; Deacon et al., 2007; Anderson et al., 2007a; Bodkin et al., 2007; Hedner et al., 2007a,b; Mathias et al., 2005; Lancel et al., 2001) indicate that the hypnotic effects of gaboxadol are maintained over long term dosing and that withdrawal phenomena after acute discontinuation of treatment are very minor.

Gaboxadol is a $GABA_A$ receptor agonist with functional selectivity for extra synaptic $GABA_A$ receptors. Experiments carried out in cell lines and *Xenopus* oocytes expressing

* Corresponding author. Department of Electrophysiology, H. Lundbeck A/S, Ottiliavej 9, DK-2500 Valby, Denmark. Tel.: +45 36433043.
E-mail address: bjeb@lundbeck.com (B. Ebert).

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different $GABA_A$ receptor combinations have shown that gaboxadol has functional selectivity for α4β3δ, α6β3δ and α4β3 containing $GABA_A$ receptors over γ2 containing $GABA_A$ receptors (Storustovu and Ebert, 2006). Since extra synaptic receptors, with the exception of α5β2/3γ2 receptors in the hippocampus, are characterised by the absence of γ2, it has been proposed that gaboxadol should be characterised as a Selective Extra Synaptic $GABA_A$ receptor Agonist, abbreviated SEGA (Nutt, 2005). Several studies have demonstrated that the pharmacological activity of gaboxadol is dependent on both the α4 and δ subunits. Chandra et al. (2006) demonstrated that gaboxadol in α4 knockout mice was unable to induce tonic currents in thalamic slices and had very little effect on motor coordination impairment, whereas the inverse was the case in wild type mice. Boehm et al. (2006) showed that in delta knockout mice the anaesthetic effect of high doses of gaboxadol was relatively shorter lasting than in wild type mice, suggesting that δ containing receptors play an important role in the activity of gaboxadol. Recently, Winsky-Sommerer et al. (2007) were able to demonstrate that the hypnotic effects of gaboxadol were highly dependent on the presence of the δ subunit. Measuring the effects of gaboxadol on sleep architecture parameters during the normal wake period, the authors demonstrated that the massive increase in slow-wave activity seen during the waking EEG after dosing with gaboxadol in wild type mice was completely abolished in δ knockout mice. These data therefore tied the activity to δ containing $GABA_A$ receptors.

As mentioned above, the clinical studies demonstrated hypnotic effects of gaboxadol maintained over months and very low incidence of withdrawal symptoms upon acute discontinuation, suggesting that the substrate for gaboxadol — probably the α4β3δ containing receptors — is not markedly affected by long term dosing. In contrast, tolerance developed acutely to the motor coordination impairment effects in mice (Chandra et al., 2006). This discrepancy between motor coordination data in animal studies and sleep data in patients led us to speculate whether different tolerance mechanisms are involved in these effects. We therefore characterised gaboxadol in a series of animal models with the aim of identifying functional consequences of down regulation of $GABA_A$ receptors activated by gaboxadol. By combining these models with CNS exposure data, we selected a dose range of gaboxadol, which yielded peak concentrations up to 3 μM (Cremers and Ebert, 2007), corresponding to doses up to 10 mg/kg/day in rats.

2. Methods

2.1. Animals

Adult, male Sprague Dawley rats (200–250 g; M&B, Denmark) pair-housed in macrolon cages (425×266×180 mm) were used in the sleep EEG studies. Male Wistar rats (150–200 g at study start; M&B, Denmark) housed 2–4 per cage (2 per cage if weighing more than 200 g) were used in the rotarod studies. The rats were house under a 12:12 hour light:dark cycle (lights on at 06:00) and had free access to standard laboratory chow and water. Room temperature (21±2 °C), relative humidity (55±5%), and air exchange (16 times per hour) were automatically controlled. Male Sprague–Dawley rats (125–150 g at study start; Charles River Breeding Laboratories, Wilmington, MA, USA) housed 3 per cage on a 12 hour light:dark cycle and with food and water ad libitum were used in the bicuculline seizure threshold studies.

2.2. Ethics

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

The protocols for the bicuculline seizure threshold studies were approved by the Tufts University School of Medicine and New England Medical Centre, Institutional Animal care and Use Committee, Boston, MA.

3. Sleep EEG studies

3.1. Surgery

Using established methodology (Vogel et al., 2002), the transmitter (TL10M3-F50-EEE implant. Data Sciences International. USA) was implanted in the peritoneum (i.p.) of the anesthetized rat. EEG leads were then placed supradurally, 2 mm anterior to bregma and 2 mm on either side of the midline for the frontal electrodes and 2 mm anterior to lambda and 2 mm on either side of the midline for the parietal electrodes. The EMG leads were placed in either side of the *musculus cervicoauricularis* and were sutured in place. The animals were allowed to recover one-week post-surgery during which an antibiotic Baytril Vet™ (enrofloxacin; 10 mg kg^{-1}) and an analgesic (Rimadyl®; carprofen; 0.1 ml per 100 g) were administered once daily subcutaneously (s.c.).

3.2. Sleep recording and scoring

Dataquest A.R.T. Gold 2.2 was used to simultaneously record EEG and EMG for 5 h immediately after dosing. The EEG data were scored as wake (W), slow-wave sleep-1 (SWS-1), slow-wave sleep-2 (SWS-2) or paradoxical sleep (PS or REM-like) according to visual analysis of EEG frequency as well as amplitude characteristics and EMG activity (Neckelmann and Ursin, 1993). The EEG recordings for each animal were visually and manually scored on the computer screen in 10 s epochs over the 5-hour recording session (3000 epochs). Data were analysed using the sleep analysis software program, Somnologica 3 (Flaga^{hf} Medical Devices, Iceland).

3.3. Drug EEG studies

Drugs were administered between 08:00 and 09:00 (lights on 06:00) and EEGs were recorded for the following 5 h. Dose–response relationships for gaboxadol (1.25, 2.5 or 5 mg/kg s.c.) and zolpidem (2.5, 5.0 and 10 mg/kg s.c.) were established using acute administration in two groups of 8 rats. In a chronic dosing

study, gaboxadol (5 mg/kg s.c. and $n=8$) and zolpidem (10 mg/kg s.c. and $n=8$) were administered once daily over a 3-week period between 08:00 and 09:00 and EEGs and EMGs were recorded before first dose (baseline) and after 1. 2 and 3 weeks of treatment. In a withdrawal study, a similar design was used and following 3 weeks of treatment with vehicle, zolpidem or gaboxadol, the animals were subjected to acute withdrawal. EEG and EMG measurements were carried out during the first two sleep periods after discontinuation of treatment.

3.4. Bicuculline seizure threshold

Rats were divided into groups of 8 animals which daily (between 10:00 and 12:00) received s.c. injections of gaboxadol (5 and 10 mg/kg), indiplon (1.25 mg/kg), zolpidem (1.25 mg/kg) or vehicle for 1 day or 28 days and were weighed twice a week to adjust dose. The mean body weights for the control group and the treatment groups were similar at the termination of experiment.

Seizure threshold against bicuculline-induced seizures in the individual groups was assessed on days 1 and 28 after drug treatment, and 24 h after the last dose. Rats were temporarily restrained while the tail vein was cannulated with a 1 ml syringe attached to a butterfly needle. To ensure vein puncture, a slight back pressure was applied to allow blood flow into the tubing. After cannulation, the animals were released from the restraint and put in a cage to allow free movement. Bicuculline was then infused at a rate of 2.0 ml/min until the first myoclonic jerk or twitch was observed. Each animal was tested once. Seizure threshold was quantified as the total dose of bicuculline (mg/kg) required to induce the first myoclinic jerk or twitch in the rat. Tolerance was defined as a decrease in the dose of bicuculline necessary to induce seizure on day 28 compared to day 1. Withdrawal was defined as an increase in the dose of bicuculline necessary to induce seizure 24 h after last treatment (day 29) compared to day 28.

3.5. Rotarod studies

Preliminary data for these experiments have previously been presented (Anderson et al., 2007b). Motor performance was assessed as previously described (Voss et al., 2003) in an automated rotarod (Rotamex 4/8, Columbus Instruments, Columbus, OH; with the 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) according to the protocols described by Voss et al. (2003). Following the final training session, the animals entered the test procedure. For all experiments, animals were divided into equal sized groups. All groups underwent an initial baseline (i.e. pre-treatment) performance assessment. In the first series of experiments, rats were dosed with 1, 3, 5, 7.9 and 10 mg/kg gaboxadol s.c. and evaluated 30, 60, 90 and 120 min post administration. In the tolerance development study, the animals were dosed and performance was assessed 30 min post administration. The animals were then returned to their home cages for 90 min, after which the challenge dose of either gaboxadol or saline was administered. Rotarod performance was assessed for the third and final time 30 minutes later.

3.6. Determination of CNS concentrations

Extracellular brain levels after s.c. administration of gaboxadol was determined as described by Cremers and Ebert (2007).

3.7. Drugs

Gaboxadol and indiplon were synthesised and zolpidem was extracted from commercially available tablets by the Department of Medical Chemistry, H. Lundbeck in Denmark. Zolpidem used in the bicuculline seizure threshold test was purchased from Sigma (St. Louis, MO) and bicuculline was purchased from Fisher Scientific (Fair Lawn, NJ).

Gaboxadol was dissolved in 0.9% NaCl and zolpidem was dissolved in 0.1 M HCl and diluted with 0.9% NaCl (final pH 5.3) in the sleep EEG and rotarod studies. Gaboxadol, indiplon and zolpidem were dissolved in saline with 2.5% dimethyl sulfoxide (DMSO) in the bicuculline seizure threshold studies. Bicuculline (0.1 mg/ml) was prepared prior to each experiment in saline with a few drops of 0.1 M HCl added. Drug solution pH was then adjusted to pH>3 with 0.1 M NaOH.

Control animals were injected with the same vehicle. All the solutions were injected subcutaneously. Injection volumes were 5 ml/kg in sleep EEG and rotarod studies and 2.5 ml/kg in the bicuculline seizure threshold studies.

3.8. Statistics

3.8.1. Sleep EEG

In the acute study, two-way repeated measures analysis of variance (ANOVA) of treatment groups was performed, followed by post-hoc analysis using the Student Newman–Keuls test. In the 3-weeks dosing study two-way repeated measures ANOVA with weeks (0 to 3) and treatment (Baseline, vehicle, gaboxadol and zolpidem) as main factors was performed followed by post-hoc analysis using the Student Newman–Keuls test to confirm significant differences between treatments. In the withdrawal study, statistical analysis was carried out by two-way repeated measures ANOVA with animal (1–8) and treatment (baseline, vehicle, gaboxadol, and zolpidem) as main factors followed by post-hoc analysis using the Student Newman–Keuls test. Differences with a p-value of less than 0.05 were considered statistically significant.

3.8.2. Bicuculline seizure threshold

The dose of bicuculline needed to induce the first myoclonic jerk or twitch was obtained for each rat and results were calculated as mean±SEM for each group. Within group data from day 1, day 28 and day 29 were analyzed using one-way ANOVA followed by Dunnett's post-hoc test. Each drug treatment group was also compared to vehicle on day 1, day 28 and day 29 using the same statistical analysis.

SigmaStat 3.00 was used for all statistical analysis (SSPS Inc., Chicago, IL). A level of $p \leq 0.05$ was considered statistically significant.

3.8.3. Calculation of level of receptor activation

CNS concentrations of Gaboxadol after dosing with 5 mg/kg and 10 mg/kg (see Cremers and Ebert, 2007) were converted

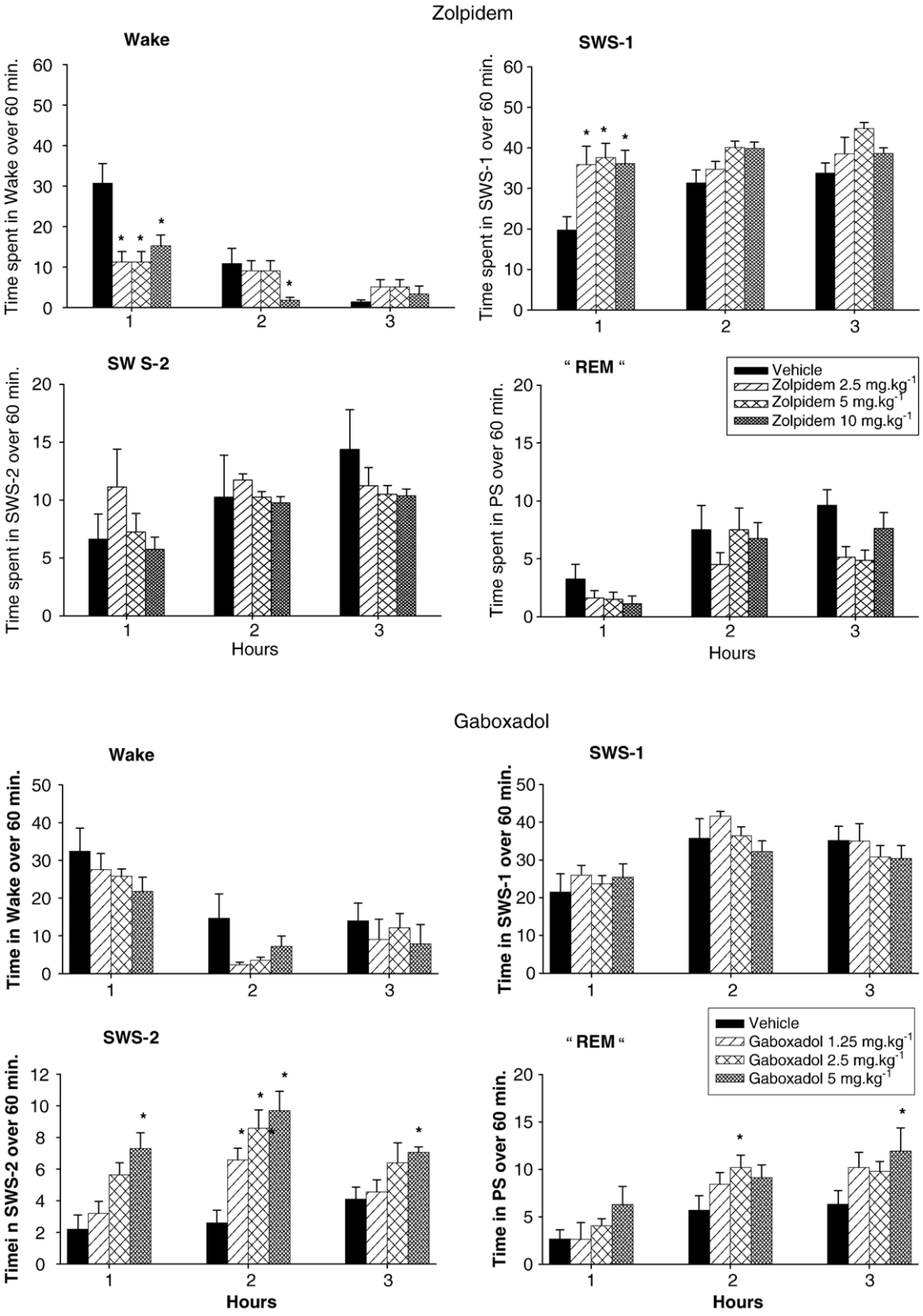

Fig. 1. Sleep parameters for rats dosed with zolpidem (top) and gaboxadol (bottom) during the first 3 h post administration. Data are represented as the mean ± SEM. *, ** and ***: Significant differences compared to vehicle ($p<0.05$; $p<0.01$ and $p<0.001$, respectively; 2 Way RM ANOVA followed by SNK test).

into levels of activation of the different $GABA_A$ receptor combinations by inserting the concentrations into the concentration response equation: $Response = Max * [Gaboxadol]^{slope} / (EC_{50}^{slope} + [Gaboxadol]^{slope})$, where Max is the maximum response, relative to that of GABA at the specific subunit combination, EC_{50} is the concentration giving half the maximum response and slope is the slope of the curve. Values for the relative maximum, EC_{50} values and slope of the curve are described in Storustovu and Ebert (2006).

4. Results

4.1. Sleep EEG

Acute dosing with gaboxadol or zolpidem produced a dose dependent effect on sleep parameters (Fig. 1). Zolpidem, 2.5 to 10 mg/kg significantly reduced the time awake for 1 h post administration. This effect was mirrored by an increase in SWS-1. At 2 h post dosing, only 10 mg/kg significantly reduced the time awake. At this time point, gaboxadol (1.25 to 5 mg/kg) numerically reduced the time awake. However, this was not statistically significant. Gaboxadol enhanced SWS-2 dose-dependently during the first 3 h post administration, and reached significant levels for all doses at 2 h post administration. Since the effects of zolpidem and gaboxadol are qualitatively different, we chose to compare the two compounds in a long term study at 10 and 5 mg/kg, respectively. At these doses the effects on time awake 2 h post administration are similar.

In the repeated dose study, gaboxadol (5 mg/kg s.c.) significantly increased total sleep time and SWS-2 during weeks 1–3 without affecting REM-like sleep or SWS-1 sleep. These data are consistent with data previously reported after 5 days of similar dosing (Lancel and Langebartels, 2000). Zolpidem significantly reduced the time awake during week 1, after which this effect disappeared. Furthermore, in zolpidem dosed rats, a reduction ($p<0.05$) in REM-like sleep was observed over the entire 3 weeks of treatment (Fig. 2).

In a separate study, withdrawal effects were quantified relative to baseline levels, measured prior to the initiation of treatment with vehicle, gaboxadol or zolpidem. Abrupt discontinuation of gaboxadol or vehicle treatment did not produce alterations in total sleep time, SWS-1, SWS-2 or REM-like, during the first (24 h withdrawal) and second (48 h withdrawal) sleeping periods (Table 1) when compared to baseline (pre-treatment). In contrast, discontinuation of zolpidem resulted in a significant reduction in total sleep time during the first sleep period (24 h withdrawal) compared to baseline [$F_{(1,6)}=8.85$; $p<0.05$]. The effect was not significant during the second sleep period (48 h withdrawal). The reduction in total sleep time during the first sleep period, was accompanied by a significant reduction in SWS-1 compared to baseline [$F_{(1,6)}=8.89$; $p<0.05$] and in REM-like sleep [$F_{(1,6)}=10.32$; $p<0.05$], of which the

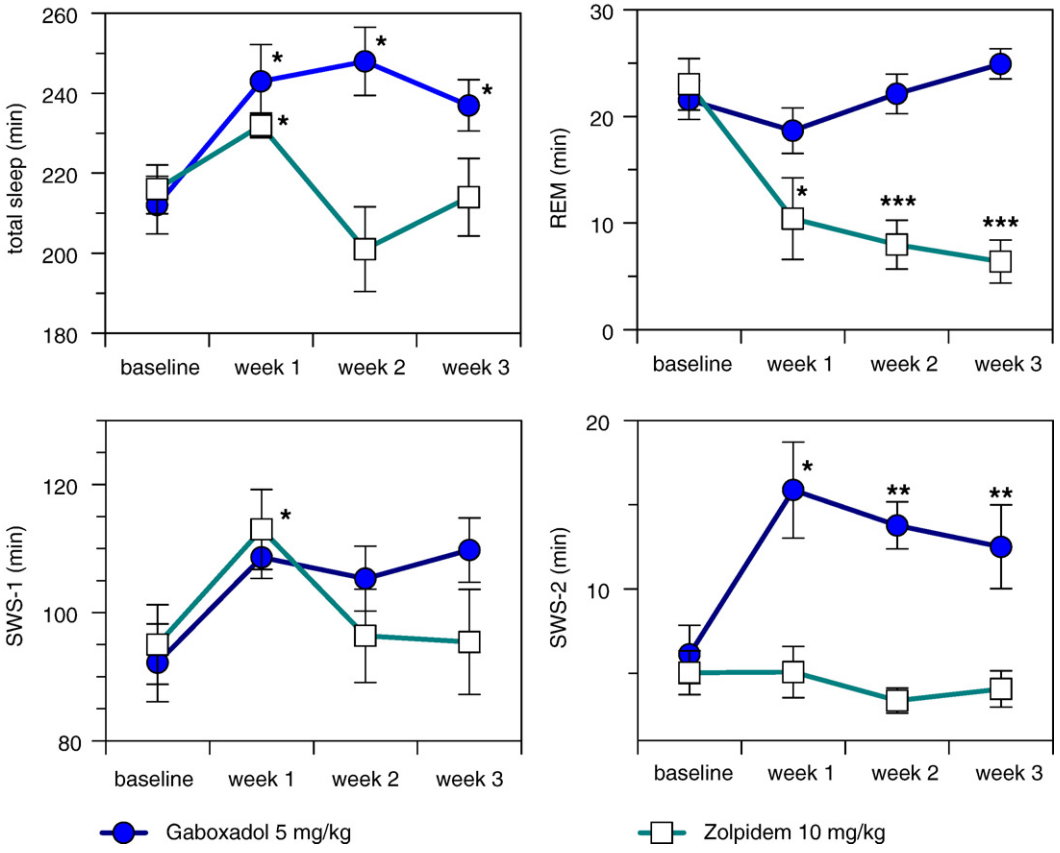

Fig. 2. Total sleep time and time spent in different sleep stages (SWS-1, SWS-2 and paradoxical sleep (REM-like)) integrated over the first 5 h post administration with gaboxadol, vehicle and zolpidem. Data are represented as the mean±SEM. *, ** and ***: Significant differences compared to baseline ($p<0.05$; $p<0.01$ and $p<0.001$, respectively; 2 Way RM ANOVA followed by SNK test).

Table 1
Amount of sleep after cessation of treatment measured in min during the first 5 h after lights on in rats being dosed daily for 3 weeks with gaboxadol, zolpidem or vehicle

	Gaboxadol			Zolpidem			Vehicle		
	Baseline	+24 h	+48 h	Baseline	+24 h	+48 h	Baseline	+24 h	+48 h
Total sleep time	218±6.6	223±8.2	226±7.4	215±6.3	165±11*	205±18	212±7.4	212±22	222±16
SWS-1	159±5.7	159±7.6	170±11	160±4.9	105±18*	122±21	159±12	154±16	175±20
SWS-2	12±2.1	15±2.8	13±2.6	8.1±1.7	8.3±1.3	8.7±1.7	10±4.2	12±3.4	7.5±1.9
"REM"	38±1.4	33±2.7	35±2.7	37±1.9	24±4.3*	28±5.0*	38±1.6	38±6.2	37±9.4

Data for baseline and sleep period 1 and 2 after acute withdrawal are shown. Values are mean±SEM. *: Significant differences compared to zolpidem baseline values ($p<0.05$; 2 Way RM ANOVA followed by SNK test).

latter remained significantly reduced during the second sleep period (48 hour withdrawal); [$F_{(1,6)}=6.07$; $p<0.05$] compared to baseline.

4.2. Seizure susceptibility

An acute dose of gaboxadol (5 and 10 mg/kg, s.c.), zolpidem (1.25 mg/kg, s.c.) or indiplon (1.25 mg/kg, s.c.) reduced the effect of bicuculline significantly (Fig. 3, left panel). With the exception of gaboxadol, 10 mg/kg/day, no apparent development of tolerance to the acute effects of bicuculline was seen following 28 days of treatment. On day 29 (24 h after the last dose) no obvious signs of hyperactivity or seizures were observed prior to the administration of bicuculline and compared to the vehicle treated animals, no difference in seizure susceptibility was detected (Fig. 3, right panel).

4.3. Rotarod

As previously described (Voss et al., 2003), gaboxadol dose-dependently impaired motor coordination. At 30 and 60 min post administration 5, 7.9 and 10 mg/kg inhibited rotarod performance significantly (Fig. 4). A time course study of 7.9 mg/kg gaboxadol demonstrated a marked attenuation of the response at 90 min and a return to baseline (i.e. non-drug treated) levels after 120 min (Fig. 5A). The attenuated gaboxadol response at 90 min is not due to tolerance but reduced drug exposure as a second dose of gaboxadol (7.9 mg/kg) or saline at this time point produce a response similar in magnitude to that of the first doses, thus ruling out that receptor desensitisation is responsible for the attenuated response.

4.4. CNS concentrations and receptor activation levels

Determination of plasma and CNS levels after s.c. administration has previously been reported (Cremers and Ebert, 2007). As illustrated in Fig. 6 (top panels), very minor differences in the CNS concentrations are obtained within the first few hours when the administered dose is increased from 5 to 10 mg/kg. This suggests that penetration of gaboxadol into the CNS is not a simple passive diffusion, but more likely a carrier mediated process. Consequences of increasing the dose are therefore primarily an extended exposure at 2–3 μM rather than an increase in peak concentration. Since most behavioural

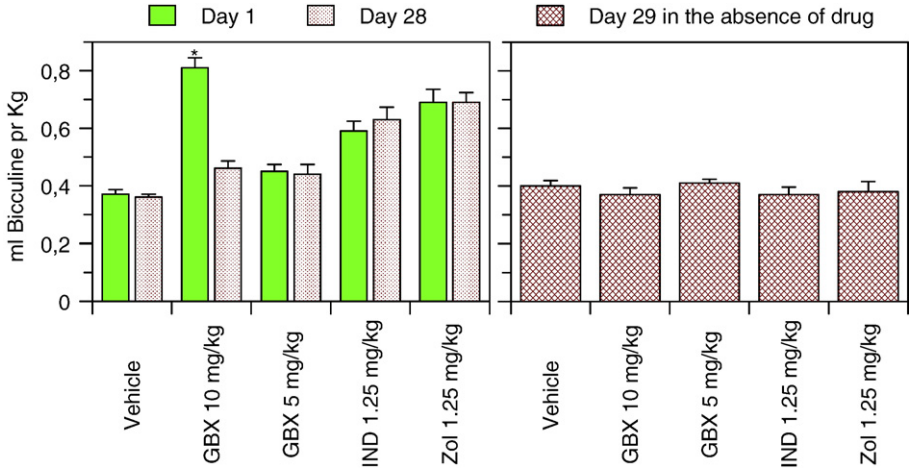

Fig. 3. Effect of chronic drug administration of gaboxadol, indiplon and zolpidem on bicuculline-induced seizures. Bars represent the mean amount of bicuculline (ml/kg)±SEM needed to induce myoclonic jerk or twitch. Left panel: Dosing with gaboxadol (5 or 10 mg/kg), indiplon (1.25 mg/kg) or zolpidem (1.25 mg/kg) at day 1 significantly increased the amount of bicuculline necessary to induce the first myoclonic jerk or twitch compared to vehicle treated animals at day 1. After 28 days of dosing, no apparent development of tolerance was observed with the exception of 10 mg/kg of gaboxadol. Significant differences ($p<0.05$) from day 1 in same treatment group are indicated by *, as determined by one-way ANOVA followed by Dunnetts post-hoc test ($n=6$). Right panel: No significant difference in seizure susceptibility after acute withdrawal from any of the three compounds compared with vehicle on day 29 or compared to day 1.

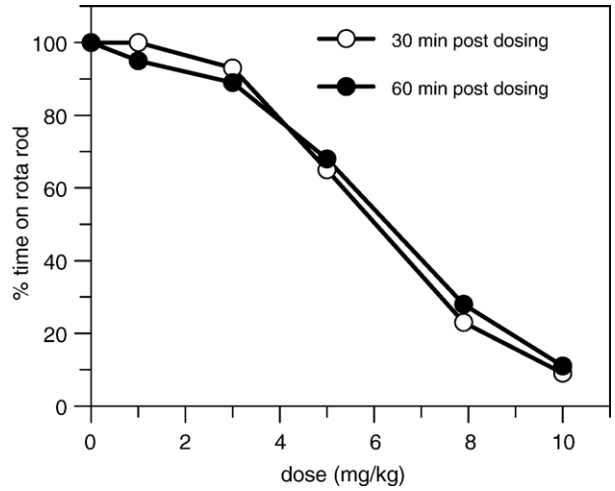

Fig. 4. Dose dependency of gaboxadol-induced impairment of rotarod performance in rats, determined at 30 and 60 min post administration. Data points represent the mean ± SEM (n = 8 rats) rotarod performance, normalised to the maximum time (90 s) spent on the rotarod in the presence of vehicle at base line condition.

studies have been carried out with doses between 5 and 10 mg/kg and only minor differences in CNS concentrations are observed within this dosage interval (Fig. 6 top panels), we chose to estimate the level of activation at α4β3δ and α6β3δ containing $GABA_A$ receptors after a subcutaneous dose of 10 mg/kg, thereby implicitly assuming that at this dose interval these receptors are responsible for the pharmacological actions of gaboxadol. As illustrated in Fig. 6 (bottom panel right), the level of activation at α4β3δ containing $GABA_A$ receptors is approximately 15% 1 h post dosing, after which it declines to approximately 5% for the following 2 h. In contrast, the level of activation of α6β3δ containing receptors is 50% 1 h post dosing after which the level of activation rapidly declines to 15% at hour two. The difference in the dynamics of the activation levels for α4β3δ and α6β3δ containing receptors is a consequence of different potencies of gaboxadol at these two receptor populations (Storustovu and Ebert, 2006). Since gaboxadol is very potent at α6β3δ containing receptors, the CNS concentrations are within the steep part of the concentration response curve and are, therefore, much more responsive to changes in concentrations. Conversely, the gaboxadol concentration response curve for the α4β3δ containing receptors is shifted further to the right and, thus, the therapeutic concentrations are within the part of the curve where the slope is low and changes in response to variations in concentrations are smaller.

By combining rotarod data, exposure data and the concentration response curves for gaboxadol at cloned human $GABA_A$ receptors, the relationship between the level of receptor activation and the time spent on the rotarod could be established. As illustrated in Fig. 7, a strong link between the activation of α4β3δ or α6β3δ containing $GABA_A$ receptors and the motor coordination impairment is present.

5. Discussion

Even though gaboxadol has been used as a standard $GABA_A$ receptor agonist tool for the last 30 years, it is not until the past few years gaboxadol has been shown to mediate its pharmacological effects at therapeutic relevant concentrations via the extra synaptically located non-gamma containing $GABA_A$ receptors. Studies in thalamic slices (Bellelli et al., 2005; Jia et al., 2005) and cortical slices (Drasbek and Jensen, 2006) have shown that the effects of gaboxadol on tonic currents, thought to be mediated via extra synaptically located α4β3δ containing $GABA_A$ receptors, appear at submicromolar concentrations and are strong and robust at 2 μM, corresponding to the maximum concentrations observed in the CNS in rats after pharmacologically active doses (Cremers

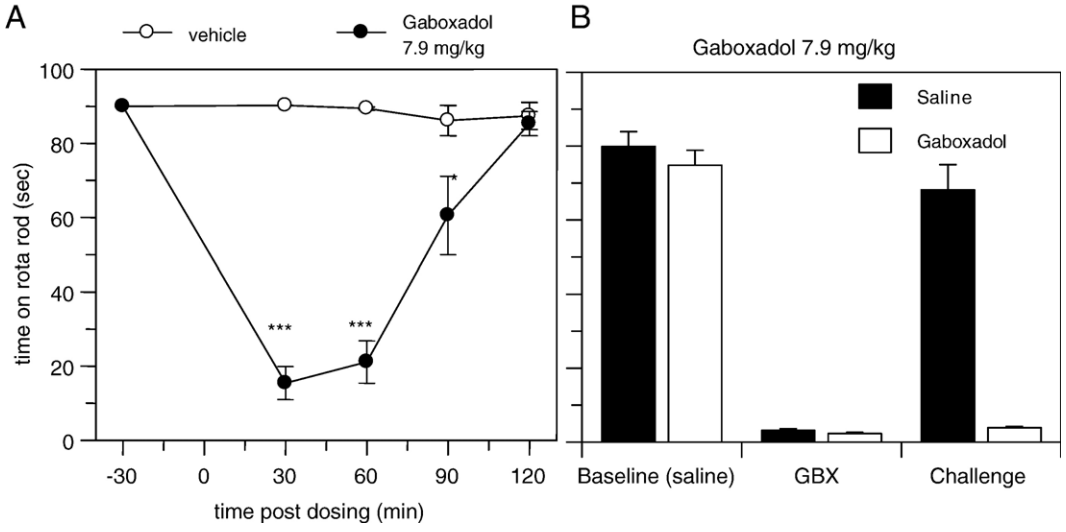

Fig. 5. A: Time course of gaboxadol-induced rotarod performance impairment in rats. Animals were dosed with either 7.9 mg/kg gaboxadol or vehicle (0.9% saline), and rotarod performance assessed at various time points post administration. Separate groups of animals were utilised for each time point to avoid any potential training effects. Values are mean ± SEM * p < 0.05; *** p < 0.001 ANOVA on ranks (pooled vehicle data vs. drug groups) followed by Dunn's post-hoc test. B: Sensitivity of rats to a second challenge of gaboxadol — as measured by rotarod performance in rats. Animals were dosed with 7.9 mg/kg or vehicle (0.9% saline), and rotarod performance was assessed 90 min post administration. A second challenge was given and the rotarod performance was evaluated 30 min later. Values are mean ± SEM (n = 8).

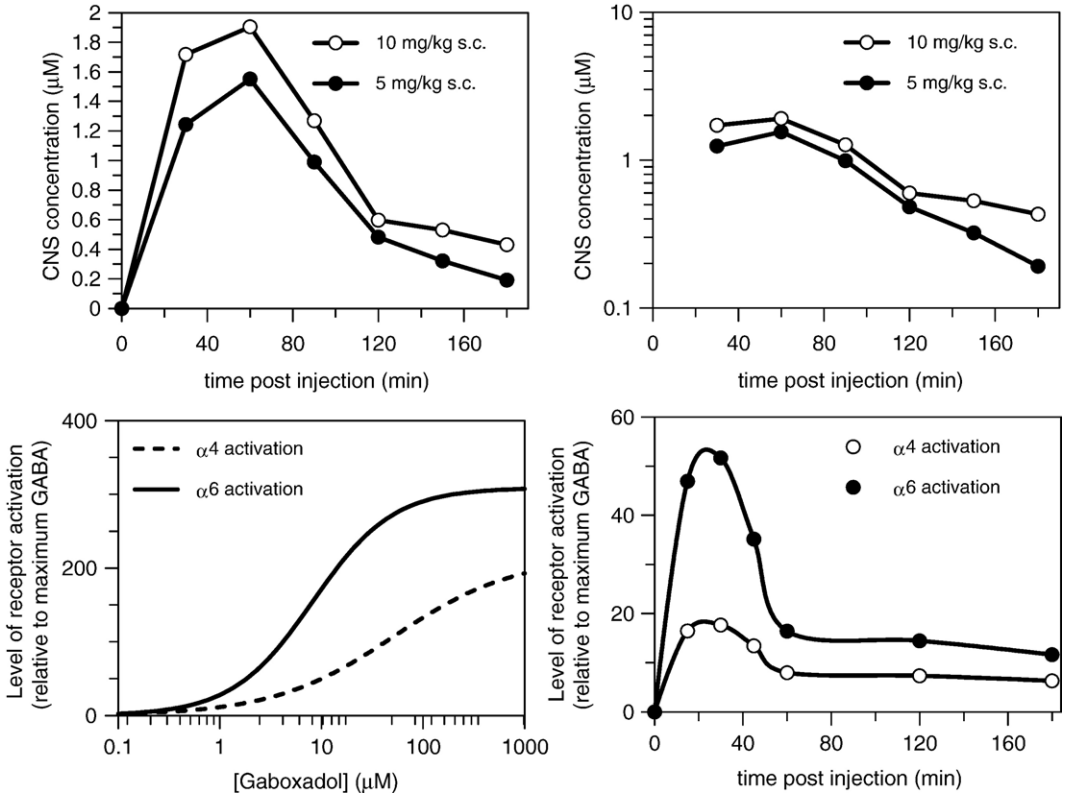

Fig. 6. Correlation between CNS concentrations and $GABA_A$ receptor activation after subcutaneous dosing with gaboxadol, Top panels: CNS concentrations plotted on linear (left) and logarithmic (right) scale (taken from Cremers and Ebert, 2007). Bottom panels: Concentration response curves for gaboxadol at α4β3δ and α6β3δ containing $GABA_A$ receptors, expressed in oocytes (left). Level of activation at α4β3δ and α6β3δ containing receptors after dosing with 10 mg/kg of gaboxadol (from Storustovu and Ebert, 2006) (right). The level of activation was calculated as described in the methods section.

and Ebert, 2007). In this concentration range, α4β3δ containing receptors are activated at approximately 20% relative to the GABA-activated maximum (Storustovu and Ebert, 2006). The low level of thalamic α4β3δ containing $GABA_A$ receptor activation by gaboxadol has been used as an explanation for the apparent lack of tolerance in clinical sleep studies. However, at the same time, a rapid apparent development of tolerance has been observed in motor coordination impairment as measured in the rotarod in both rats (Voss et al., 2003) and mice (Chandra et al., 2006). These earlier data raised the question of whether tolerance in fact does develop at extra synaptic receptors at therapeutic relevant concentrations of gaboxadol. In order to address this question, we carried out a series of experiments, where tolerance development previously has been demonstrated with $GABA_A$ receptor modulating compounds. Sleep EEG measurement of zolpidem and gaboxadol in rats during a 3 week daily treatment paradigm demonstrated no apparent tolerance development with gaboxadol, whereas tolerance to zolpidem was observed after two weeks of treatment measured as total sleep time and SWS-1. The lack of tolerance development to gaboxadol's effect on sleep is also reflected in the lack of withdrawal phenomena after acute discontinuation of treatment. These findings suggest that chronic dosing with gaboxadol is not be associated with compensatory mechanisms, e.g. up regulation of excitatory neurotransmitter systems or down regulation of $GABA_A$ receptors. However, none of these possibilities have been addressed experimentally in the current study. A possible explanation for the lack of tolerance development is that gaboxadol, at peak concentrations, elicits approximately 20% of the maximum GABA response at α4β3δ containing receptors, which may be below the level needed for

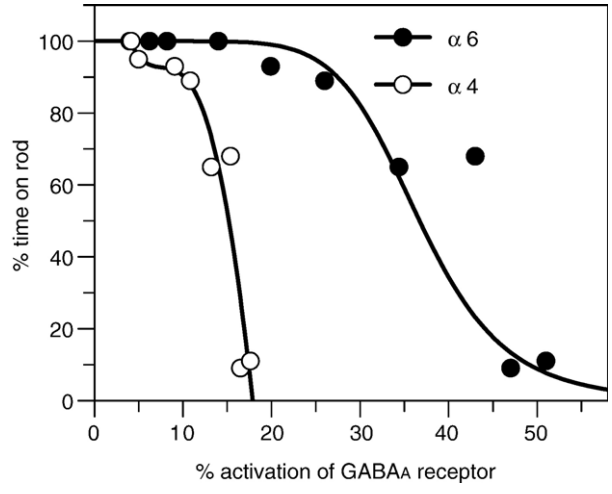

Fig. 7. Correlation between time spent on the rotarod and the level of activation by gaboxadol of α4β3δ and α6β3δ containing $GABA_A$ receptors. Using data, where time on rotarod, dose in mg/kg and CNS concentration were determined, the level of activation as described in the Methods section was calculated. Values for time on rotarod are normalised to maximum time spent on rotarod in vehicle treated groups (90 s).

initiating compensatory mechanisms. It is interesting to note that zolpidem, which has a similar half life in the rat but mediates its effects via synaptically located γ2 containing $GABA_A$ receptors, displays a rapid development of tolerance and shows significant discontinuation effects on the first day of withdrawal. These data are in line with the mechanism of action theories suggested by Saper et al. (2005), where the main site of action of gaboxadol on sleep is mediated in the ventral lateral preoptic area of the thalamus. The data are also in agreement with data from Belleli et al. (2005), who demonstrated that the tonic currents induced by gaboxadol in thalamic slices containing the ventral basal part do not desensitise. Conversely, synaptic currents that are modulated by benzodiazepines are insensitive to modulation by gaboxadol at therapeutic levels. This phenomenon is likely associated with the high level of receptor activation necessary for producing these effects (e.g. see Wafford and Ebert, 2006) and may reflect the sensitivity of the sleep system to minor changes in the balance of excitatory/inhibitory input. However, when animals dosed once daily with gaboxadol, zolpidem or indiplon for 4 weeks and subjected to acute withdrawal and subsequent challenge with the convulsive bicuculline, no functional indication of receptor down regulation is seen. Although a change in a functional end point like drug-induced seizures most likely reflects larger changes in $GABA_A$ receptor density than does modulation of an endogenous sleep mechanism, these data indicate that no dramatic change in the excitatory/inhibitor balance was induced. Previous studies with benzodiazepine receptor agonists have clearly shown tolerance development and significant discontinuation effects (e.g. Hutchinson et al., 1996; Malcolm, 2003), but the majority of these studies have been conducted with long acting agonists or continuous benzodiazepine exposure using implanted pellets, minipumps or via food or drinking water. Constant exposure, therefore, seems to be a prerequisite for demonstrating strong discontinuation effects of benzodiazepine agonists (e.g. seizure susceptibility), whereas more therapeutically relevant dose schemes confirm the clinical experience with zolpidem and a lack of severe withdrawal effects.

In the rotarod studies a rapid attenuation of the response to gaboxadol is demonstrated, resulting in a complete normalisation 90–120 min post administration. Motor coordination impairment, which can be a consequence of both altered spinal reflexes and cerebellar GABAergic activity, is associated with activation of cerebellar GABAergic neurons primarily containing 6β3δ and to some extent α1β2/33δ, α1α6β2/3δ, α6β2/3γ2 and α1α6β2/3γ2 subunits. Studies by Yamashita et al. (2006) and Zheng et al. (1994) have shown that at low GABA concentrations, the response in cerebellar granule cells does not desensitise, whereas, at higher concentrations corresponding to EC_{30} or above, desensitisation does occur. However, the degree of desensitisation is never complete, resulting in a steady state current irrespective of receptor activation level. The attenuated response to gaboxadol might therefore be a consequence of some degree of receptor desensitisation of α6 containing $GABA_A$ receptors in the cerebellum. However, when rats were re-challenged, the impairment in motor coordination is comparable to the effect of the first dose. It is, therefore, highly unlikely that the attenuated response after 90 to 120 min is a consequence of desensitisation or down regulation of receptors.

The relation between CNS concentrations of gaboxadol the concentration response curves of GABAA receptors expressed in *Xenopus* oocytes and the rotarod data indicated that a high level of α6β3δ containing $GABA_A$ receptor activation took place at the beginning of the rotarod experiment and then declined from 60 min post dosing. At 90 and 120 min post administration, the levels of receptor activation at α6β3δ fell to approximately 20–30% and were similar to those obtained with the inactive doses of 1 and 3 mg/kg. In contrast, the level of α4β3δ receptor activation ranged between 10 and 20% during the first 3 h, irrespective of dose and time. These data suggest that the rapid decline in the level of α6β3δ receptor activation may be the explanation to the attenuated response. This, however, does not rule out that an effect mediated via α4β3δ containing receptors is so dramatic, that minor changes in the level of receptor activation may be responsible for the fading in the effects on motor coordination. This could be interpreted as a very steep nearly all-or-none receptor activation response curve as illustrated in Fig. 7. However, behavioural studies have not indicated such a steep dose–response relationship (Grech and Balster, 1997; McDonald et al., 2007; Michelsen et al., 2007), suggesting that α4β3δ containing $GABA_A$ receptors only contribute marginally to the motor coordination impairment effects seen with gaboxadol. This is supported by the fact that α4β3δ containing $GABA_A$ receptors are not expressed in cerebellar areas controlling motor coordination (Pirker et al., 2000). However, Chandra et al. (2006) compared wild type mice with α4 knockout mice and found that these receptors strongly contribute to the motor coordination impairment effects of gaboxadol. According to our analysis (Fig. 7) activation levels at α6β3δ containing receptors above 25% 6 knockout mice (Chandra et al., 2006) show a reduced motor coordination impairment effect of GABAergic compounds similar to α4 knockout mice. It is therefore premature to conclude which of these two GABAergic receptor populations that determine the effects of gaboxadol. It may well be, that although cerebellar receptors are the most important determinant of motor coordination impairment under normal circumstances, α4β3δ receptors contribute to this effect in a subtle manner. Furthermore, as has been shown in several types of knockout mice, it may well be that compensatory mechanisms in the form of up regulation of other $GABA_A$ receptors may occur, thereby making these mice more susceptible to the effects of gaboxadol.

In conclusion, although previous data have suggested that tolerance to the acute motor coordination effects of gaboxadol develops rapidly, the fading response can be explained purely on the basis of exposure and activation parameters. This strongly highlights the value of combining behavioural pharmacology, CNS pharmacokinetics in the CNS and relevant electrophysiology. The presented data also highlight the importance of conducting pharmacological studies under therapeutically relevant conditions. Although dogma tells us that long term dosing in animals with benzodiazepines leads to severe discontinuation symptoms like reduced seizure threshold, this

is not the case when short acting compounds are administered once daily at relevant doses.

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