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Plasma and CNS concentrations of Gaboxadol in rats following subcutaneous administration

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Abstract

Gaboxadol has been suggested to be a selective extrasynaptic $GABA_A$ receptor agonist. However, there is little information on Gaboxadol concentrations in the central nervous system (CNS) at therapeutically relevant doses. In order to investigate this, rats were injected subcutaneously with Gaboxadol and plasma and CNS concentrations were determined using the dynamic-no-net-flux and ultraslow microdialysis methods. Results using the 2 methods were similar and showed that Gaboxadol rapidly entered the brain and that peak CNS concentrations after 2.5, 5 and 10 mg/kg were in the range of 0.7 to 3 μM. Furthermore, a very short half-life (28 min) in both plasma and CNS was observed. It is concluded that concentrations of Gaboxadol in the CNS are in a range, which are likely to activate only extrasynaptic (nongamma subunit containing) $GABA_A$ receptors.

Index Terms: Gaboxadol; Microdialysis; GABA; *In vivo*; (Rat)

1. Introduction

GABA is the principal inhibitory neurotransmitter in the central nervous system. Through interaction with the ionotropic $GABA_A$ receptors GABA mediates diverse phenomena as anxiolysis, muscle relaxation and sleep. The $GABA_A$ receptor is a pentameric transmembrane protein consisting of five subunits that form a central anion channel. Several different $GABA_A$ receptor subunits have been identified and subdivided into eight distinct groups, many with several variants: α1–6, β1–3 γ1–3, ρ1–3, δ, ε, π, and θ (Bateson, 2004; Sieghart et al., 1999; Whiting et al., 1999). Only certain combinations of different subunits appear to occur in the human brain. Most receptor subtypes appear to comprise two α, two β and one γ subunit. The δ subunit may replace the γ subunit, and preferentially co-assembles with either α_4 or with α_6 subunits. Many other combinations have also been identified and it seems likely that others may yet be detected (Bateson, 2004; Whiting et al., 1999). Receptors containing the α_1-subunit constitute 60% of the receptor population, and are widely distributed in the brain, whereas the α_4, α_5, and α_6 subunits are more discretely expressed, confined largely to the thalamus (α_4), hippocampus (α_4,α_5), and cerebellum (α_6) (Bateson, 2004; Whiting et al., 1999).

Gaboxadol is a $GABA_A$ receptor agonist, which currently is in phase III clinical development as a hypnotic. Over the last years several preclinical studies have demonstrated an unexpected functional receptor selectivity profile of Gaboxadol. Gaboxadol strongly activates α4βδ containing $GABA_A$ receptors expressed in different cell types and *Xenopus* oocytes (Storustovu and Ebert, 2006; Brown et al., 2002; Storustovu and Ebert 2003) leading to an apparent functional selectivity for these receptors over other $GABA_A$ receptors. Since delta-containing $GABA_A$ receptors seem to be expressed exclusively outside the synapse (Nusser et al., 1998), Gaboxadol has been classified as the first in a novel pharmacological class of compounds: Selective Extrasynaptic $GABA_A$ receptor Agonists, abbreviated SEGA (Nutt, 2005). This classification is confirmed by data from electrophysiological studies in slices

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from hippocampus, cortical, and thalamic preparations (Jia et al., 2005; Drasbek and Jensen, 2006; Belleli et al., 2005; Chandra et al., 2006). In these studies, low μM concentrations of Gaboxadol activated extrasynaptic receptors, leading to increased tonic current, whereas no effect on synaptic communication was seen. Additionally, in transgenic mice lacking the delta subunit, Boehm et al. (2006) demonstrated that the anaesthetic effects of Gaboxadol were significantly reduced, whereas *in vitro* effects were only marginally affected. Rota rod studies in rats (Voss et al., 2003) have shown no synergistic interactions between Gaboxadol and benzodiazepine receptor agonists, or Gaboxadol and ethanol, suggesting that benzodiazepine receptor agonists mediate their effects via receptors different from those activated by Gaboxadol. Furthermore, in the same study, no cross-tolerance between Gaboxadol and zolpidem was seen, again pointing towards different target receptor population receptors. In drug discrimination studies in primates (McMahon and France, 2005) and rats (Ator, 1971; Ator and Griffiths, 1986; Nielsen et al., 1987), benzodiazepines did not generalise to Gaboxadol, whereas Gaboxadol showed partial generalisation to pentobarbital (5 or 10 mg/kg i.p. training dose) in two studies (Ator and Griffiths, 1986; Grech and Balster, 1993). The only compound to which Gaboxadol has fully generalised is the $GABA_A$ agonist, muscimol (1 mg/kg i.p. training dose; Grech and Balster, 1997; Jones and Balster, 1998), again supporting the notion that Gaboxadol and benzodiazepines mediate their effects via different receptor populations.

However, studies with synaptic receptors expressed in *Xenopus* oocytes or cell lines have repeatedly shown that Gaboxadol, at concentrations higher than those that activate extrasynaptic receptors, interacts synergistically with benzodiazepines. This effect is also observed for all other $GABA_A$ receptor agonists (e.g. Wong and Iversen, 1985). In order to address if the apparent functional selectivity of Gaboxadol in *in vitro* studies can be extended to *in vivo* by relevant concentrations in the central nervous system (CNS), determination of plasma and CNS concentrations is important. In the current study we have determined concentrations of Gaboxadol in the plasma (total and unbound fraction) and CNS after subcutaneous dosing.

2. Materials and methods

2.1. Animals

Male Wistar rats (280–350 g BWT; Harlan, Zeist, The Netherlands) were used for the experiments. Rats were individually housed in plastic cages (30×30×40 cm) and had *ad libitum* access to food and water. Experiments were carried out in accordance with the declarations of Helsinki and were approved by the Animal Care Committee of the College of Mathematics and Natural Science, University of Groningen.

2.2. Surgery

Rats were anesthetized using isoflurane (2%, 400 ml/min N_2O, 400 ml/min O_2). Lidocain (10% w/v) was used for local anesthesia. Each animal to be used for microdialysis experiments was placed into a stereotaxic frame (Kopf instruments, USA), and I-shaped probes for dynamic no-net-flux (Hospal NM 6/4 membrane, 4 mm exposed surface, Brainlink, The Netherlands), or I-shaped MetaQuant probes for Ultra-slow microdialysis (PES membrane, 4 mm exposed surface, Brainlink, The Netherlands) were inserted into the medial prefrontal cortex (mPFC) using the rat brain atlas of Paxinos and Watson (1982). Coordinates for the tips of the probes were: mPFC (AP=3.3 mm from bregma, $L=-0.8$ mm, $V=5.0$ mm from dura). The probes were then fixed to the skull with dental cement and a screw. Flunixin (1 mg/kg s.c.) was administered as post-operative analgesic. Experiments were carried out 48 h after surgery. After completion of the experiments the rats were sacrificed. The brains were removed and cured in paraformaldehyde solution (4% w/v). The positioning of each probe was verified histologically according to Paxinos and Watson (1982), by making coronal sections of the brain.

In a separate group of rats, each animal to be used for blood sampling experiments had a 10-cm segment of Silicone tubing (0.64 mm ID; 0.94 mm OD) inserted into the isolated right jugular vein. The catheter was exteriorized through an incision on top of the head, where it was fitted onto a metal elbow. The catheter was kept patent by filling it with a 40% polyvinylpyrrolidone solution containing 500 IE/ml heparin. The end of the venous catheter was fixed in position with dental acrylic cement and anchored to the skull with two stainless steel screws. Flunixin (1 mg/kg s.c.) was administered as post-operative analgesic. Experiments were carried out after allowing the animals at least 48 h to recover from surgery.

2.3. Experiments with dynamic no-net-flux microdialysis

On the day of the experiment, rats were connected with flexible PEEK tubing to microperfusion pumps (CMA 102), and the dialysis probes were perfused with 0, 1 and 10 μM Gaboxadol dissolved in a Ringer buffer containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$, at a flow rate of 1.5 μl/min. Before starting the experiment, the conditions in the probes were allowed to stabilize for 1 h after the flow had been initiated. Microdialysis samples were collected at 15 min intervals into empty mini-vials by means of an automated fraction collector (CMA 142), and stored at −80 °C until analysed. Dialysis samples were collected for 240 min after s.c. administration of either 2.5, 5, or 10 mg/kg (free base) Gaboxadol, and three more vials were filled with the perfusate itself.

2.4. Experiments with ultraslow flow microdialysis

On the day of the experiment, rats were connected with flexible PEEK tubing to microperfusion pumps (CMA 102). The dialysis probes were perfused with 0.2% (w/v) bovine serum albumin dissolved into a filtered Ringer buffer containing 147 mM NaCl, 3.0 mM KCl, 1.2 mM $CaCl_2$, and 1.2 mM $MgCl_2$, at a flow rate of 0.1 μl/ min. The same Ringer buffer (but without albumin) was run through the dilution inlet of the

probes at a flow rate of 0.9 μl/ min. Before starting the experiment, the conditions in the probes were allowed to stabilize for 1 h after the flow had been initiated. Microdialysis samples were collected at 30 min intervals into empty mini-vials by means of an automated fraction collector (CMA 142), and stored at −80 °C until analysed. Dialysis samples were collected between $t=0$ and $t=390$ min. At $t=15$ min the rats received an s.c. injection of 2.5, 5, or 10 mg/kg (free base) Gaboxadol. Appropriate flow of the ultraslow microdialysis was verified by determining the weight gain of the vials, and by continuing the collection of diluent for 1 more hour after the ultraslow flow had been turned off.

2.5. Experiments with systemic blood sampling

On the day of the experiments, rats were connected with flexible Tygon tubing to automated blood sampling equipment (AccuSamplers, Dilab, Sweden). Blood samples (250 μl) were collected from conscious unrestrained animals at $t=0$, 0.25, 0.5, 1, 2, 3, 5, 7, 9, 12, 24 h after s.c. administration of either 2.5, 5, or 10 mg/kg (free base) Gaboxadol. After each sample had been taken, loss of blood volume was compensated by infusion of an equal amount of heparinized (15 IE/ml) saline. Periodic infusions of 30 μl of this saline solution also kept the catheters patent.

Blood samples (250 μl) were collected into microtubes containing 5 μl heparinized (500 IE/ml) saline, and immediately centrifuged at 14,000 rpm for 10 min to recover plasma. Plasma was separated into two aliquots. Free Fraction: 75 μl of plasma was centrifuged in spin tubes (10 kDa membrane cutoff) at 14,000 rpm at 4 °C for 60 min before being stored at −80 °C. Total Fraction: the remaining 90 μl plasma was stored at −80 °C without filtration procedures.

2.6. Determination of Gaboxadol in dialysates and plasma

Concentrations of Gaboxadol in dialysate and plasma samples were determined by HPLC with tandem mass spectrometry (MS/MS) detection. All samples were assayed after extraction and purification with acetonitrile-based mobile phase solution. Gaboxadol was quantitated by internal standard (to correct for extraction and ionization efficiencies) and external standard methods.

2.6.1. Extraction procedure

Samples (15 μl dNNF microdialysate, or 30 μl MQ microdialysate and plasma) were mixed with 150 μl internal standard solution (66.7 ng/mL d4-Gaboxadol in acetonitrile) and centrifuged at 14,000 rpm for 5 min. Solvent from the supernatant was aspirated at 45 °C under a gentle stream of N_2 gas. Gaboxadol extract was then re-dissolved in 100 μl redissolving fluid (70% acetonitrile, 30% methanol), ready to be injected onto the HPLC.

2.6.2. Separation by HPLC

Samples (75 μl) were injected onto the HPLC column by an automated sample injector (PerkinElmer Instruments, series 200). Chromatographic separation was performed on a reverse-phase 150×2.00 mm (5 μm) analytical column (Asahipak NH2P, Phenomenex), held at a temperature of 30 °C. The mobile phase (isocratic) consisted of 70% (v/v) 5 mM ammonium acetate in ultra-purified water, and 30% (v/v) acetonitrile, pH=7. Mobile phase was run through the system at a flow rate of 0.400 ml/min by an HPLC pump (PerkinElmer Instruments, series 200 micro pump). For experiments using the API 3000 in conjunction with the ionics source (300 °C), the mobile phase was set at 0.3 ml/min, followed by post-column addition of 0.025% NH4OH in ACN at a flowrate of 0.1 ml/ min.

2.6.3. LC-MS/MS detection

The LC/MS analyses were performed using an API 4000 MS/MS system consisting of an API 4000 MS/MS detector and a Turbo Ion Spray interface, or an API 3000 with an Ionics ion source (Applied Biosystems, the Netherlands and MS vision resp.).

The acquisitions on API 4000 were performed in negative ionization mode, with ion spray voltage set at −4.5 kV, the nebulizer gas pressure at 50 psig (on a SCIEX scale 0–90) with a probe temperature of 425 °C. The instrument was operated in multiple-reaction-monitoring (MRM) mode for detection of Gaboxadol (precursor 139.1 Da, product 110.1 Da), and d4-Gaboxadol (precursor 143.0 Da, product 112.2 Da). The CE was −16 eV, DP −50, and CXP at −7 eV and the collision gas (nitrogen) pressure was held at 3 (on a SCIEX scale of 0–12). Data were calibrated and quantitated using the Analyst™ data system (Applied Biosystem, version 1.2). Setting for the API 3000 with ionics source (300 °C) was similar except that CE was set at −17, DP −7, and CXP at −6 eV. Sensitivity of both systems was 0.5 ng/ml per injection.

3. Results

3.1. Plasma

Following a s.c. injection, Gaboxadol was rapidly absorbed. As illustrated in Fig. 1, peak concentrations were obtained within 30 min after injection. Clear dose proportionality in peak concentrations and an elimination constant suggesting a one-compartment system was observed. Analysis of free plasma concentrations revealed that protein binding was below 15% in the first 3 h after administration (13.8+ 8.0%, $n=4$).

Similar data were seen in the CNS (Figs. 1 and 2) concentration profile, and a close to 1:1 ratio between CNS and total plasma concentrations was observed after 60 min and during the following 2 h, after which concentrations of Gaboxadol in CNS fell below detection limits.

3.2. CNS concentrations: dynamic-no-net-flux

The *in vivo* recovery as estimated with the dynamic-no-net-flux method showed considerable variation between subjects and experimental groups. Recoveries varied from 3.5 to

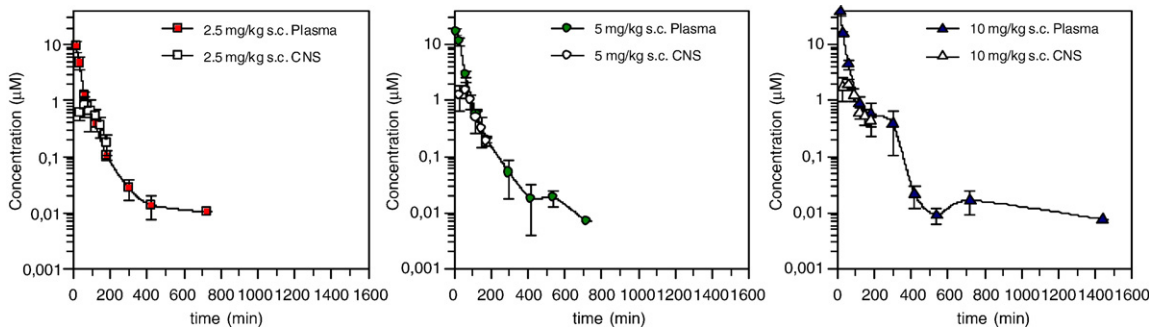

Fig. 1. Concentrations of Gaboxadol in plasma and CNS after s.c. administration. Extracellular levels of Gaboxadol in the CNS after subcutaneous administration quantified with the modified ultraslow microdialysis method. Closed symbols: Plasma concentrations and open symbols: CNS concentrations.

20% indicative of the high variation, which is commonly observed with this method. Brain levels of Gaboxadol were observed to reach 150 (1.1 µM), 350 (2.5 µM) and 400 (2.9 µM) ng/ml, respectively, after administration of 2.5, 5 and 10 mg/kg of subcutaneous administration of Gaboxadol (Fig. 2). Levels declined to below detection limits 3 h after dosing.

3.3. CNS concentrations: ultraslow microdialysis

Microdialysis was performed at 100 nl/min, which enabled the recovery to approach 100% (Cremers et al., 2001). *In vivo* retrodialysis of 10 µM Gaboxadol yielded 100% recovery ($n=3$) and *in vitro* recovery of 10 µM Gaboxadol was 98.2± 7.8% ($n=3$). Gaboxadol penetrated the brain in a dose dependent manner after subcutaneous administration. Injection of 2.5 mg/kg Gaboxadol induced brain levels of approximately 100 ng/ml (0.7 µM) after 60 min, declining to about 50 ng/ml (0.4 µM) at 3 h (Fig. 2). Administration of 5 mg/kg induced brain levels of approximately 200 ng/ml (1.4 µM) after 60 min, showing levels equal to the 2.5 mg/kg at 3 h after dosing. After administration of the highest dose of 10 mg/kg Gaboxadol, brain levels of approximately 300 ng/ml (2.1 µM) were observed 1 h after administration, followed by a slow elimination after 2 h (Fig. 2).

Observed brain levels tended to be marginally lower when quantified using ultraslow dialysis versus dynamic-no-net-flux.

4. Discussion

Until recently, Gaboxadol has been regarded as a standard $GABA_A$ receptor agonist, which penetrates the blood brain barrier. Several behavioural studies have clearly indicated that Gaboxadol must enter the brain (e.g. Grognet et al., 1983; Chandra et al., 2006) and as such it was assumed that the compound would activate all types of $GABA_A$ receptors. Since several preclinical studies now strongly suggest that the main site of action for Gaboxadol is extrasynaptically located $GABA_A$ receptors (see Introduction), it is important to establish if therapeutic concentrations are within a range that can explain both the pharmacodynamic effects and the lack of interaction with benzodiazepines. Therefore, the present study represents an attempt to evaluate whether the SEGA concept (Nutt, 2005) was consistent with exposure data obtained in animals. Following an s.c. injection of 2.5 to 10 mg/kg, which previously has been shown to act hypnotic (Vogel et al., 2003), the plasma concentration of Gaboxadol rapidly reached high micro molar levels, after which it was redistributed and elimination took place. Fitting of the data after 1 h to a mono exponential decay showed that the decline in concentration in both CNS and plasma was independent of the dose and followed the same kinetics with an elimination constant (ke) of 1.47±0.07 corresponding to a half life in both CNS and plasma of 28 min.

Quantification of extracellular levels of exogenous compounds in the brain using microdialysis is a complex procedure (De Lange et al., 2000). Estimation of levels *in vivo* is not only dependent on membrane properties, but also depends on various

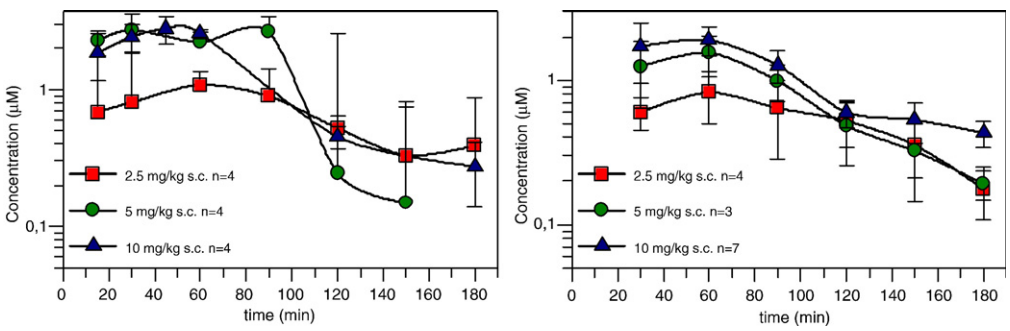

Fig. 2. Extracellular levels of Gaboxadol in the CNS after s.c. administration as quantitated using the dynamic-no-net-flux method (left) and the modified ultraslow microdialysis method (right).

processes in the brain. This composite of factors such as brain vasomotricity, permeability, and metabolism determines how much of the actual compound reaches the probe, and hence all contribute to the *in vivo recovery* (De Lange et al., 2000). In the current study we used 2 methods to quantify Gaboxadol in the brain, the dynamic-no-net-flux (Olsen and Justice, 1993) and ultraslow microdialysis (Cremers et al., 2001). Although the methods significantly differ in their approach, they yielded similar results, confirming their reciprocal validity.

Using both methods, we observed that Gaboxadol penetrates the brain extensively. After the initial redistribution of Gaboxadol in plasma and CNS, the concentrations and elimination from these two compartments seemed to follow similar parameters. Since no particular transporters of Gaboxadol over the blood brain barrier have been identified, it is likely that passive diffusion alone can account for the CNS pharmacokinetics. The protein binding that was observed in the present study was below 15%. This is similar to the binding in humans (Lund et al., 2006). When plasma levels are corrected for protein binding, the data further confirm the passive penetration of Gaboxadol in the brain, which is apparent when free plasma and brain levels reach unity after an initial equilibration stage (De Lange et al., 2000). In all, these data, indicate that Gaboxadol readily penetrates the brain and suggest that the concentration determined in the brain is a direct reflection of the concentration available for receptor interaction.

In line with this notion are *in vitro* data describing the distribution of Gaboxadol and muscimol over bio membranes or octanol–water at physiological pH. In these studies the distribution of Gaboxadol into the lipophilic phase was significantly higher than muscimol and this correlated with the fraction of unionized molecule, which is much higher for Gaboxadol than for muscimol hence facilitates its penetration in the brain (Krogsgaard-Larsen et al., 1985).

Published pharmacokinetic studies performed in humans indicate peak plasma concentrations of Gaboxadol after oral dosing of 15 mg are obtained after approximately 30–60 min and are in the 1–3 μM range (Lund et al., 2006; Shadle et al., 2006). Since peak plasma concentrations are smaller in humans than in rats, it is most likely that the CNS concentrations in humans will be at or below the low micro molar concentrations.

Care should be taken while extrapolating plasma and brain levels from rodents to humans. Arguably an important factor inducing discrepancies between rodents and humans is differences in protein binding between species. As mentioned above the observed protein binding in the present study and clinical studies were similar (Lund et al., 2006), hence facilitating comparison of species. An additional liability for extrapolation between species is active carrier mechanisms within the brain (De Lange et al., 2000). However, no such active transport has been shown for Gaboxadol. Additionally, the observed unity between free plasma and brain levels during the elimination phase further corroborates the notion for passive brain transport of Gaboxadol.

As illustrated in Figs. 1 and 2, following an s.c. application of Gaboxadol 10 mg/kg, the compound rapidly entered the brain where peak concentrations in the 2–5 μM range were observed. Based on functional data obtained in oocytes (Storustovu and Ebert, 2006) these initial high concentrations are activating human $\alpha4\beta\delta$ containing $GABA_A$ receptors, whereas $\alpha1$, $\alpha2$ and $\alpha5$ containing synaptic receptors are activated at very low levels. Since most behavioural animal studies are conducted within the first 30 min after drug administration, one would predict that even at the peak concentration of Gaboxadol little or no synergistic interaction between Gaboxadol and benzodiazepine receptor agonists or Gaboxadol and ethanol should be seen. This is indeed in agreement with currently available data from the rat *rota rod* studies (Voss et al., 2003). The reason for this lack of interaction may well be due to the strong activity of Gaboxadol at extrasynaptic receptors. At high CNS concentrations, a very large current is carried through the delta-containing receptors, whereas the synaptic component relative to the overall current is small (Belleli et al., 2005). Therefore some low level activation of non-extrasynaptic $GABA_A$ receptors may be present immediately after an s.c. application with Gaboxadol, but any potential functional significance may be masked by the large extrasynaptic current.

However, in delta unit knock out mice, the consequences of dosing with Gaboxadol are more ambiguous. Winsky-Sommerer and Tobler (2006) report that delta knock out mice do not respond with the normally increased amount of slow wave activity in wake or nonREM sleep (Vyazovskiy et al., 2005) and therefore interpret their data as indicative of delta-containing $GABA_A$ receptors as being responsible for the hypnotic effects of Gaboxadol. Boehm et al. (2006) have characterised the anaesthetic effects of Gaboxadol in delta knock out mice. In these experiments, a very high dose (30 mg/kg) of Gaboxadol inhibits the writhing reflex for much shorter time than in wild type mice. These data may indicate that delta-containing $GABA_A$ receptors are contributing to the anaesthetic effects of Gaboxadol, but it also highlights that the functional selectivity of Gaboxadol only appears at low micro molar concentrations. At higher concentrations other $GABA_A$ receptor populations will contribute significantly to the pharmacological activity of Gaboxadol.

The CNS concentrations of Gaboxadol are so low that only extrasynaptic receptors are activated and only to a very low extent. In most studies dealing with CNS concentrations, the issue of the actual concentration at the synapse remains open. Since extrasynaptic receptors are located outside the synapse, there is direct access to the receptor from the extracellular fluid and the receptor active concentration can therefore be estimated to be similar to the CNS concentration as quantified by microdialysis.

It has previously been described that long term dosing with Gaboxadol does not lead to tolerance to hypnotic effects in rats (Vogel et al., 2003). There is therefore no indication of functional down regulation of the target receptors in relation to the hypnotic effects of Gaboxadol during long term dosing. It is tempting to speculate that the combination of rapid kinetics and low level of receptor activation prevents the development of tolerance. However, a rapid development of tolerance to the motor coordination impairment measured in the rota rod has been observed after s.c. dosing, highlighting the heterogeneity in responses to Gaboxadol (Chandra et al., 2006). Motor coordination in rats is most likely mediated via extrasynaptic $\alpha6\beta3\delta$ containing receptors in the cerebellum. As shown by Storustovu and Ebert (2006), Gaboxadol is very potent at this receptor combination.

Subcutaneous dosing of 2.5 or 5 mg/kg leads to CNS concentrations, and subsequently receptor activation of α6β3δ containing receptors, close to 100%, which may result in desensitization and the development of tolerance to the locomotor effects. The absence of tolerance to the hypnotic effects of Gaboxadol may therefore be due to the relatively low activation of α4β3δ containing receptors.

In conclusion, the present study demonstrates that in rats Gaboxadol rapidly penetrates the brain after systemic administration and that levels of Gaboxadol in brain are equal to levels in plasma after an initial distribution phase. The concentrations obtained lie within a range, which are consistent with a selective activation of extrasynaptic $GABA_A$ receptors. The dissociation between the very short half-life in brain and the longer lasting effects on sleep remains enigmatic.

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