Pharmacoresistance in epilepsy : modelling and prediction of disease progression

Liefaard, C.

Chapter VII

Decreased efficacy of $GABA_A$ receptor modulation by midazolam in the kainate model of temporal lobe epilepsy

Lia C. Liefaard[a], Rou-Afza F. Gunput[a], Meindert Danhof[a], Rob A. Voskuyl [ab]

[a]Division of Pharmacology, LACDR, Leiden University, Leiden, The Netherlands
[b]SEIN – Epilepsy Institutes of The Netherlands Foundation, Heemstede, The Netherlands

Epilepsia, 48(7):1378–1387, 2007

Summary

Purpose The objective of this investigation was to quantitatively characterise time dependent changes in midazolam efficacy in the silent period after induction of status epilepticus in rats. The changes in midazolam efficacy were correlated to changes in *ex vivo* $GABA_A$ receptor expression.
Methods Midazolam efficacy was quantified by pharmacokinetic-pharmacodynamic (PK-PD) modelling using the β-frequency of the EEG as PD endpoint. Two PK-PD experiments were performed in each animal: the first experiment before and the second experiment at either day 4 or day 14 after status epilepticus. Status epilepticus was induced by repetitive intraperitoneal injections with kainate. $GABA_A$ receptor expression was determined by *ex vivo* autoradiography with [^{3}H]flumazenil.
Results The concentration *versus* EEG effect relationship of midazolam was successfully described by the sigmoidal E_{max}-model. The maximal effect on the β-frequency of the EEG (E_{max}) was reduced to 51.6 ± 35.6% and 25.8 ± 33.7% of the original value at 4 and 14 days after induction of status epilepticus. The *ex vivo* study with [^{3}H]flumazenil showed that the observed reductions in E_{max} were parallelled by a reduction in $GABA_A$ receptor density.
Conclusions The efficacy of midazolam is decreased in the silent period after status epilepticus, which can be partly accounted for by a reduction in $GABA_A$ receptor density.

7.1 Introduction

Despite the introduction of a considerable number of new antiepileptic drugs over the last decades,[1] the fraction of patients that continue to have drug-resistant seizures has remained stable at about 30%.[2] Pharmacoresistance occurs in nearly all types of seizures and epileptic syndromes, but mesial temporal lobe epilepsy (mTLE) in particular is among the most difficult-to-treat and common types of epilepsy.[3] The universal occurrence of pharmacoresistance over all epilepsy types indicates that several mechanisms are involved. In recent years, two theories have been put forward: (a) over expression of multidrug-transporters at the blood-brain barrier, limiting access of antiepileptic drugs to the brain and (b) alterations of drug targets in the brain leading to reduction or

elimination of drug responsiveness, in particular in voltage-gated Na^+ channels and $GABA_A$ receptors.[4,5]

There is much evidence for alterations in $GABA_A$ receptors in mTLE, resulting in decreased GABAergic effects.[6] Both in human and in animal studies a decrease in benzodiazepine binding has been shown. For instance, using *ex vivo* studies with the benzodiazepine antagonist [^{3}H]flumazenil in brain tissue of patients, the binding of flumazenil has been shown to be decreased in different hippocampal areas.[7] The same results were found *in vivo* in a PET study with [^{11}C]flumazenil in TLE patients.[8] In both studies the decrease in $GABA_A$ receptor density is more pronounced than the neuronal cell loss, suggesting not only neurodegeneration of GABAergic neurons, but also a disappearance of $GABA_A$ receptors.[8]

Experimental studies in post-status epilepticus models of mTLE, such as the pilocarpine model or the kainate model, have also shown that epileptogenesis is associated with profound changes in $GABA_A$ receptors. In the kainic acid induced post-status epilepticus model binding of [^{3}H]flunitrazepam or expression of $GABA_A$ receptor mRNA is altered in different brain regions at 12 or 24 hours after onset of status epilepticus.[9,10] Furthermore, rapid and extensive changes in subunit expression have been reported, parallelled by changes in *in vitro* pharmacology.[11] This may well explain the rapid reduction in benzodiazepine response that has been observed *in vivo*. Termination of pilocarpine-induced status epilepticus by diazepam or phenobarbital, two drugs which act by modulation of GABAergic inhibition, appeared to be dependent on time after induction of status epilepticus: diazepam is very effective in terminating status epilepticus when injected only a few minutes after the first stage III motor seizure, whereas it is less effective at later time points.[12]

While it is likely that the rapid changes in $GABA_A$ receptors during or directly after status epilepticus contribute to the concomitant reduction in responsiveness to diazepam and phenobarbital, it remains to be demonstrated that these changes also underlie long-term pharmacoresistance. Spontaneous seizures emerging some weeks after status epilepticus induction in animals are not totally unresponsive to drugs acting on GABAergic inhibition.[13] Thus, it is conceivable that the acute changes during status epilepticus are, at least to some extent, transient. Furthermore, other mechanisms may emerge at later stages and contribute to pharmacoresistance as well. To determine whether the events at the molecular and cellular level and the alterations in *in vivo* drug response are causally related, it is necessary to determine the efficacy and potency of drugs in quantitative terms and in relation to the time course of the epileptogenic process. Such an analysis can also indicate whether a loss in responsiveness to antiepileptic drugs is absolute or can (in principle) be overcome by increasing the dose.

In the present study, we hypothesised that the rapid decrease in benzodiazepine-responsiveness during status epilepticus is long-lasting and progressive, extends into the subsequent latent period and is causally related to changes in $GABA_A$ receptor mediated inhibition. Therefore, the efficacy of midazolam before the induction of status

epilepticus by kainic acid and during the silent period after status epilepticus in the same rats was compared to study the impact of epileptogenesis on the GABA-modulating effect of benzodiazepines. Experiments were performed during the latent period, because several studies have shown that GABAergic inhibition is affected already during the latent period,[9,10] and alterations in GABAergic inhibition during the latent period may cause development of pharmacoresistance during later stages of epileptogenesis. To investigate whether there were progressive alterations in GABAergic inhibition after induction of status epilepticus, experiments were performed at day 4 and day 14 after treatment with kainic acid.

Spontaneous seizure activity does not occur before induction of status epilepticus and at best only sporadically during the latent period after status epilepticus. Monitoring electrographic or overt seizure activity is therefore no option for use as pharmacodynamic endpoint to assess the efficacy of midazolam, or to test development of epilepsy. In this study, the increase in the total amplitude of the β-frequency band of the EEG was used as a measure for the facilitation of $GABA_A$ receptor mediated inhibition. The β-activity of the EEG is an attractive pharmacodynamic measure, because it can be continuously measured.[14] Studies by Mandema *et al* have shown that the changes in β-activity and *in vivo* potency correlate well with the *in vitro* efficacy and affinity of a whole spectrum of benzodiazepines, from full agonists to inverse agonists.[15,16] Furthermore, both potency and maximal EEG effect for different benzodiazepines have been shown to closely correlate with their ability to suppress seizure activity.[15]

The data were quantitatively analysed by means of population pharmacokinetic-pharmacodynamic (PK-PD) modelling. In this approach serial blood samples are collected and changes in β-activity are recorded simultaneously, to obtain concentration-time and EEG effect-time data for each animal. In this way, possible effects of alterations in pharmacokinetics on the EEG-effects are accounted for. Another advantage of this approach is that the data of the whole population are simultaneously analysed, while taking into account inter-individual variability in parameter values by using stochastic models.[17] This makes it possible to assess the influence of specific characteristics, such as induction of status epilepticus or *ex vivo* flumazenil binding on individual parameters of the PK-PD model by including these characteristics as covariates.[18,19]

Finally, using *ex vivo* autoradiography with [^{3}H]flumazenil it was investigated whether changes in $GABA_A$ receptor modulating effects of midazolam after induction of status epilepticus were caused by a decrease in $GABA_A$ receptor expression.

7.2 Methods

7.2.1 Animals

Adult male Sprague Dawley rats (Harlan, Horst, The Netherlands) were used, weighing 200–250 g at arrival. The animals were housed individually, at a constant temperature of 21 °C and a 12 hour light/dark cycle, in which the lights were switched on at 8 AM. Food (standard rat/mouse chow: SRM-A, Hope Farms, Woerden, The Netherlands) and water were available *ad libitum*.

Animal procedures were performed in accordance with Dutch laws on animal experimentation. All experiments were approved by the Ethics Committee for Animal Experiments of the Leiden University.

7.2.2 Experimental setup

The primary goal of this study was to compare the efficacy of midazolam before and after status epilepticus. To control for the effect of repeated midazolam administration, a control group was included which did not experience status epilepticus. To distinguish the various treatments, the total of 26 rats was divided into kainic acid-treated animals and controls (see table 7.1). A schematic representation of the experimental setup can be found in figure 7.1.

Cortical electrodes for EEG recording were implanted under general anaesthesia with 0.25 mg/kg fentanyl citrate and 8 mg/kg fluanisone (Hypnorm, Janssen Pharmaceutica, Tilburg, The Netherlands) and 18 mg/kg sodiumpentobarbital (Nembutal, Ceva Sante Animale, Maassluis, The Netherlands). Both anaesthetics were administered intraperitoneally. Two stainless steel electrodes (1.2 mm diameter) were implanted stereotactically over the frontoparietal neocortex at a position 1.0 mm posterior to bregma and 3.5 mm left and right of the sutura saggitalis. The reference electrode was placed 2.5 mm posterior to lambda. The electrode wires were attached to a connector (MS 363, Plastics One, Roanoke, VA, USA) and the assembly was secured to the skull using dental acrylic cement. The animals were allowed one week for recovery.

Two days before the first PK-PD-experiment with midazolam, indwelling cannulas were implanted in the left femoral artery for the serial collection of blood samples, and in the right jugular vein for drug administration. This surgical procedure was performed under anaesthesia with ketamine base (Ketalar, Parke-Davis, Hoofddorp, The Netherlands, 1 μg/g body weight, subcutaneously) and medetomidine hydrochloride (Domitor, Pfizer, Capelle a/d IJssel, The Netherlands, 0.1 μg/g body weight, intramuscular).

Two PK-PD experiments with midazolam were performed in each animal: the first experiment was performed 2 days before induction of status epilepticus, and the second experiment at either day 4 or day 14 after status epilepticus. To control for effects of repeated administration of midazolam, control groups were included, in which the rats received saline injections, instead

Table 7.1: Number of animals for the different treatment groups.

	Days before/after treatment with kainic acid/vehicle[a]			
Treatment group	**−2**	**4**	**14**	*n*
kainic acid-treated[b]	x	**X**		7
kainic acid-treated[b]	x		**X**	8
control[c]	x	x		5
control[c]	x		x	6

[a] Experiment with midazolam after treatment with kainic acid is indicated with "**X**", experiment with midazolam before treatment with kainic acid/vehicle, or after treatment with vehicle is indicated with "x".
[b] Animals of the kainic acid-treated groups were treated with kainic acid to induce status epilepticus.
[c] The control groups were treated with saline instead of kainic acid.

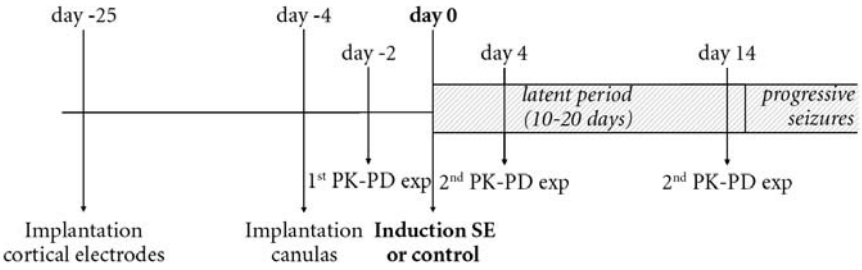

Figure 7.1: Schematic overview of experimental design. Two PK-PD experiments with midazolam were performed in each animal: the first experiment before, and the second experiment at either day 4 or day 14 after status epilepticus (SE) or control. Status epilepticus was induced by repetitive intraperitoneal injections with kainic acid. Control animals received saline injections.

of kainic acid. In these rats, the efficacy of midazolam was determined at the same time points. In table 7.1 the number of animals in each group is given. After the second midazolam experiment the brains of the rats were isolated, and used for *ex vivo* binding studies with flumazenil.

7.2.3 Induction of status epilepticus

Status epilepticus was induced by repeated intraperitoneal injections with kainic acid according to the method described by Hellier *et al.*[20] Briefly, kainic acid, dissolved in saline, was injected intraperitoneally once per hour, until class IV/V motor seizures (according to Racine's scale) occurred, or a total amount 30 mg/kg kainic acid was administered. Typically, 3 injections with kainic acid (first injection 10 mg/kg, all other injections were 5 mg/kg) were needed. The control rats received intraperitoneal injections with saline.

7.2.4 PK-PD experiment with midazolam

All PK-PD experiments were started between 9:00 and 9:30 AM to standardise influences of circadian rhythms. The rats were placed in a rotating drum to control the level of vigilance, thereby avoiding the interference of sleep patterns. During the experiments, the rats were deprived of food and water for the duration of the experiment (max 210 minutes). The signal from the EEG lead on the left hemisphere, with reference to the lead on the cerebellum, was amplified using a DAM50 differential amplifier (World Precision Instruments, Sarasota, FL, USA), with the gain set at 10 k, the low pass filter set at 0.1 Hz and the high pass filter set at 100 Hz. After band-pass filtering through a high pass filter with 70 dB/octave roll off set at 30 Hz (Department of Physiology, Leiden University, Leiden, The Netherlands), the signal was fed into a 80486 personal computer using a BNC/16 interface (Viewdac, Keithley, USA) with a sample frequency of 256 Hz. Matlab (The Mathworks, Natick, MA) was used for off-line fast Fourier transformation at an epoch length of 10 seconds to obtain the total amplitude in the β-band (11.5–30 Hz). To reduce the amount of data, amplitudes were averaged over predefined time intervals. The data of the baseline were averaged per 10 minutes, and from start of midazolam infusion the following intervals were used: 1 interval of 1 minute, 7 intervals of 2 minutes, 27 intervals of 5 minutes, and 3 intervals of 10 minutes, subsequently.

After recording of EEG baseline for 30 minutes, a 2 minute zero-order intravenous infusion of 10 mg/kg midazolam (Bufa, Uitgeest, The Netherlands) was administered to the conscious and freely moving rat using an infusion pump (Harvard Apparatus, South Natick, MA, USA). Midazolam was dissolved in equimolar hydrochloric acid. Serial arterial blood samples of 50–200 μl (larger samples at the end of the experiment) were taken at t = 0, 2.5, 5, 7.5, 10, 15, 20, 30, 55, 90, 115, 150, and 175 minutes after start of infusion. The total volume of withdrawn blood samples was 1.1 ml during each experiment. The withdrawn volume of blood for each sample was compensated for by saline. Blood samples were heparinised and centrifuged at 5000 rpm for 15 minutes for plasma collection. The plasma samples were stored at −20 °C until HPLC analysis.

7.2.5 Drug analysis in plasma

The plasma concentrations of midazolam were determined by HPLC using UV detection (222 nm) according to Mandema *et al*[21] with the modifications described by Visser *et al.*[22]

7.2.6 Ex vivo binding studies with [^{3}H]flumazenil

Directly after the second PK-PD experiment with midazolam, the rats were deeply anaesthetised with sodiumpentobarbital (Nembutal, Ceva Sante Animale, Maassluis, The Netherlands) and perfused through the ascending aorta with phosphate buffered saline (PBS) followed by a 4% formaldehyde (Merck, Germany) solution in PBS. After one night *in situ* post fixation at 4 °C, the brains were dissected and overnight cryoprotected in 30% sucrose in 0.1 M phosphate buffer (pH 7.4). Finally the brains were frozen in isopentane (−30 °C) and stored at −80 °C until further processing.

The brains were horizontally sliced with a sliding microtome in sections of 40 μm, which were collected in DMSO (Baker, Deventer, The Netherlands) / glycerin (Sigma, Zwijndrecht, The Netherlands), and stored at −20 °C. For autoradiography with [^{3}H]flumazenil, sections at the midlevel of the hippocampus (about 5–5.5 mm ventral from brain surface) were mounted on Superfrost Plus slides (Menzel-Glaser, Merck, Amsterdam, The Netherlands) and air-dried. All procedures were carried out at room temperature. The sections were pre-incubated 3 times for 10 minutes in 100 ml ice cold 50 mM Tris-HCl, pH 7.4 (USB, Bakker, The Netherlands), to remove endogenous GABA. The sections were air dried and incubated for 80 minutes in Tris-HCl (USB, Bakker, The Netherlands) buffer containing 23.4 nM [^{3}H]flumazenil (NEN life science/ Perkin Elmer, The Netherlands). After incubation the sections were washed 2 times for 2 minutes in ice cold Tris-HCl, pH 7.4 (USB, Bakker, The Netherlands), followed by a dip in ice cold distilled water to remove residual salts. Non-specific binding was determined in the presence of 25 μM unlabelled flumazenil (Sigma, Zwijndrecht, The Netherlands). The film used for imaging was a Kodak Biomax MS Scientific imaging film (Kodak, Rochester, NY).

To quantify $GABA_A$ receptor binding in the cortex, the optical density of the radiolabel in a small area (about 50000 μm^2) was quantified using an Olympus image analysis system (Paes BV, Zoeterwoude, The Netherlands). The mean greyness measured was corrected for the non-specific binding and background. The mean binding in the control animals was set at 100%, and the binding in each individual kainic acid-treated rat was calculated as percentage of the mean binding in the control animals (B_{FMZ}).

7.2.7 Data analysis

The data was analysed by means of non-linear mixed effect (population) modelling using NONMEM (version V, NONMEM project group, University of California, San Francisco,

USA). A more detailed background of population modelling is described elsewhere.[17] Shortly, population analysis results in estimates of three types of parameters: (a) the structural model parameters, which are average values for the population of the parameters describing the dose-concentration-effect relationships, (b) inter-individual (IIV) and inter-occasion (IOV) variances and covariances of the structural model parameters, and (c) residual variance, which is associated with measurement/intra-individual error.[17] By taking into account IIV and IOV, individual *post hoc* estimates of the structural parameters are obtained.[23] Furthermore, the standard error (SE) of the estimation of all parameters and variances is given. The coefficient of variation in %, defined as the SE as percentage of the parameter estimate,[23] is used as measure of the precision of parameter estimation.

To analyse the PK-PD relationship of midazolam, a step-wise approach was applied. The first step consisted of the modelling of the PK of midazolam. As the precision of the PK parameter estimates (and thereby also the precision of the PD parameter estimates) increases with the number of animals, we took advantage of the possibility to expand the database for the population PK analysis with the data of 24 animals from a similar experiment. The added animals were treated identically in all respects as compared to the control animals, except that they received cortical stimulation before the PK-PD experiment. Before accepting the result we confirmed that the addition of extra animals did not alter the values of the PK parameter estimates. The concentration-time data were described with the use of a three-compartment model, resulting in estimates of midazolam clearance (CL), intercompartmental clearances (Q_2, and Q_3), and volumes of distribution of the different compartments (V_1, V_2, and V_3). IIV and IOV in any parameter were described with an exponential variance model. The residual error was assumed to be proportional to the plasma concentration.

In the second step the relation between plasma concentration (C) and EEG-effect (E) was described by the sigmoidal E_{max}-equation:[24]

$$E = E_0 + \frac{E_{max} \cdot C^{n_H}}{EC_{50}^{n_H} + C^{n_H}} \tag{7.1}$$

in which E_0 represents the baseline EEG activity, E_{max} the maximal response to midazolam, EC_{50} the concentration at half-maximal response, and n_H the Hill factor. The concentrations (C) were predicted for each individual rat using the *post hoc* estimates of the PK-parameters. IIV and IOV in any parameter were described by an additive variance model. The residual error of the PD-analysis was characterised with an additive error model.

The effect of kainic acid-treatment on the midazolam EEG-effect was incorporated in the model as follows:

$$E_{max,exp2} = \alpha \cdot E_{max,exp1} \tag{7.2}$$

in which $E_{max,exp1}$ represents the individual *post hoc* estimate of the E_{max} of the first (pre-status epilepticus) midazolam-experiment, and α a factor to describe the fractional difference between E_{max} of the two midazolam-experiments (pre-status epilepticus and post-status epilepticus experiment). The remaining IIV in $E_{max,exp2}$ was described by an additive variance model. It appeared that there was a relation between the decrease in E_{max} after status epilepticus and the amount of *ex vivo* binding of [^{3}H]flumazenil, which was best described by an exponential function. Therefore, the relation between the estimates of $E_{max,exp1}$ and $E_{max,exp2}$ was further refined by incorporating the measured *ex vivo* binding of [^{3}H]flumazenil:

$$E_{max,exp2} = e^{\beta \cdot B_{FMZ}} \cdot \frac{E_{max,exp1}}{100} \tag{7.3}$$

in which B_{FMZ} represents the percentage of binding of [^{3}H]flumazenil as measured *ex vivo* (see "*Ex vivo* binding studies with [^{3}H]flumazenil"). Also in this case the remaining IIV in $E_{max,exp2}$ was described by an additive variance model.

7.2.8 Statistical analysis

Goodness of fit of both PK and PD-analyses were evaluated by visual inspection of two types of diagnostic plots. Firstly, the data of individual observations *versus* individual or population predictions should be randomly distributed around the line of identity. Secondly, the weighted residuals versus time or population predictions should be randomly distributed around zero.

The decision to include IIV or IOV for any parameter was based on (a) whether the variability was significantly different from zero, (b) whether inclusion did not affect estimation of any other parameter, and (c) whether inclusion improved goodness of fit.

Statistical analysis was performed using paired *t*-test for comparison of *post hoc* estimates of first and second experiments. Unpaired *t*-test with Welch correction was used to compare *post hoc* parameters of experiments performed at day 4 and at day 14 after induction of status epilepticus. Statistical tests were performed using InStat version 3.0 for Windows (GraphPad, San Diego, USA).

7.3 Results

7.3.1 Pharmacokinetic analysis of midazolam concentration-time profiles

Based on goodness of fit criteria, the concentration-time profiles were best described by a three-compartment model. All data were simultaneously analysed to obtain most accurate and precise estimates of the PK-parameters, which are given in table 7.2. To study whether there were differences in PK between the different treatment groups, the

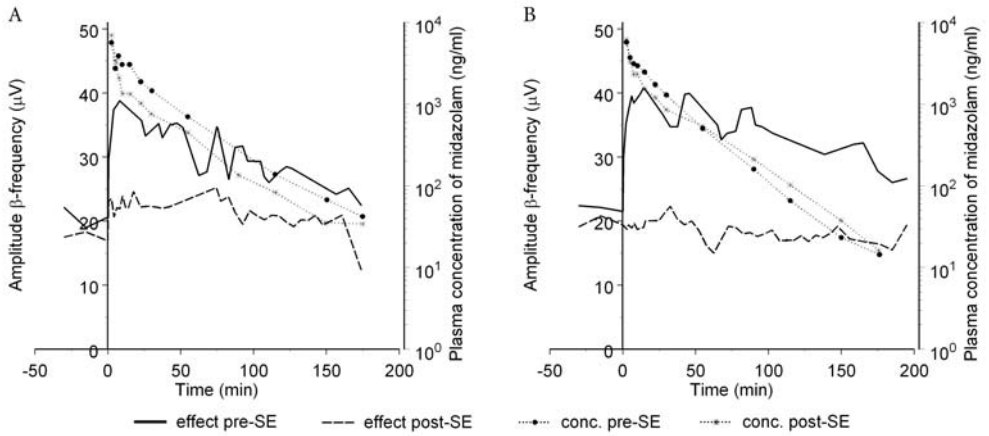

Figure 7.2: Typical examples of midazolam plasma-concentration, and EEG-effect *versus* time curves before (solid lines) and after induction of status epilepticus with kainic acid (dashed lines). In panel A the second experiment was performed at day 4 after status epilepticus, in panel B at day 14 after status epilepticus. At *t* = 0 min 10 mg/kg midazolam was intravenously infused in 2 minutes.

mean values of CL and V_2 were compared (table 7.3). This shows that the estimates of CL on the two different occasions within the same rats were identical, indicating that repeating midazolam administration or kainic acid treatment did not affect CL. Typical examples of midazolam concentration-time profiles, obtained before, and at 4 or 14 days after induction of status epilepticus by kainic acid are shown in figure 7.2, illustrating that there are indeed no appreciable differences in pharmacokinetics of midazolam between these experiments.

Using individual *post hoc* estimates of the structural PK-parameters, concentrations at the PD-observation time points were predicted. Subsequently, it was investigated whether there was hysteresis (delay) between concentration and effect. Based on the plots of estimated concentration *versus* observed effect, there was no significant hysteresis,

Table 7.2: Population estimates of PK-parameters and variabilities.

Parameter	Value[a]	95% confidence interval
Structural parameters		
CL (ml/min)	24.6 ± 1.1 (4.4%)	22.5–26.7
Q_2 (ml/min)	84.0 ± 5.7 (6.7%)	72.9–95.1
Q_3 (ml/min)	1.8 ± 0.4 (22.7%)	1.0–2.6
V_1 (ml)	215 ± 19.4 (9.0%)	177–253
V_2 (ml)	499 ± 21.4 (4.3%)	457–541
V_3 (ml)	127 ± 26.8 (21.1%)	74.5–180
Inter-individual & inter-occasion variability[b]		
ω^2_{CL} (IIV)	0.040 ± 0.017 (44.0%)	0.0054–0.074
ω^2_{CL} (IOV)	0.029 ± 0.0079 (27.4%)	0.012–0.044
$\omega^2_{V_2}$	0.068 ± 0.019 (27.5%)	0.031–0.10
ω^2_{CL,V_2} (covariance)	0.041 ± 0.017 (41.3%)	0.0078–0.074
Residual error[c]		
σ^2_{PK}	0.051 ± 0.0073 (14.3%)	0.037–0.065

[a] Parameter estimates ± SE. Between brackets coefficient of variation in %.

[b] Inter-individual variability (IIV) was significantly different from zero for CL and V_2, signifying that these parameters can be different between individuals of the population. As all animals underwent two PK-PD experiments with midazolam (before and at either 4 or 14 days after induction of status epilepticus), there might be differences between the two experiments. Therefore inter-occasion variability (IOV) was tested, which was found to be significantly different from zero for CL, which may indicate *a*) a random variation, *b*) an effect of repeating midazolam administration, *c*) an effect of inducing status epilepticus, or *d*) a combination of these factors.

[c] The residual error, which accounts for any measurement/intra-individual error, is assumed to be proportional to the midazolam concentration in blood.

Table 7.3: Mean values of CL and V_2 for the different treatment groups.

Treatment group	CL **(ml/min)**[a b] **1st experiment**	CL **(ml/min)**[a b] **2nd experiment**	V_2 (ml)[a c]	**day 2nd experiment**
kainic acid-treated	30.9 ± 6.4	27.9 ± 3.3	529 ± 71.7	4
kainic acid-treated	30.8 ± 2.6	24.8 ± 5.4	603 ± 95.9	14
control	29.5 ± 4.7	31.2 ± 6.2	533 ± 44.8	4
control	27.3 ± 0.5	26.2 ± 2.3	504 ± 130	14

[a] Mean of *post hoc* estimates ± SD.
[b] Both inter-individual (IIV) and inter-occasion variability (IOV) for CL were significantly greater than zero, resulting in individual *post hoc* estimates of CL for each individual animal, and for each occasion (1st (before status epilepticus) or 2nd (afer status epilepticus)). There were no significant differences between experimental groups or occasions (1st *versus* 2nd experiment, $p > 0.05$).
[c] For V_2 only IIV was significantly different from zero, thus individual *post hoc* estimates of V_2 only for each individual animal could be calculated. There were no significant differences between experimental groups ($p > 0.05$).

implying that plasma and brain concentrations were at equilibrium immediately. Therefore, predicted plasma concentrations were directly linked to the observed effects in further analysis.

7.3.2 PK-PD analysis of midazolam effects: control experiments

The relation between the PK and PD of midazolam was described by the sigmoidal E_{max}-model. The data of all first PK-PD experiments and of the second experiments of the controls were analysed simultaneously. This analysis resulted in parameter estimates as presented in table 7.4. Table 7.5 shows that there were no significant differences between the mean values of the *post hoc* estimates of the different treatment groups, and the different occasions. Thus, the repeating midazolam administration in itself did not affect E_{max}.

7.3.3 Alterations in effect of midazolam after induction of status epilepticus

All kainic acid-treated animals underwent a status epilepticus of at least three hours upon treatment with kainic acid, during which the rats exhibited intensive class IV/V motor seizures. In figure 7.2 typical examples of EEG-effect *versus* time curves of midazolam before and after induction of status epilepticus are given. This figure clearly shows that the effect of midazolam is substantially reduced at 4 days after status epilepticus, and completely absent at day 14 after status epilepticus.

The data were analysed with the sigmoidal E_{max}-model. First, it was checked which parameter of the concentration-effect relation was affected by induction of status epilepticus. As only E_{max} was altered as result of kainic acid-treatment, the values of EC_{50} and n_H were fixed to the values as estimated from the control experiments. To take into

Table 7.4: Population estimates of PD-parameters and variabilities of control experiments.

Parameter	Value[a]	95% confidence interval
Structural parameters		
E_{max} (μV)	10.4 ± 0.6 (5.8%)	9.2–11.6
EC_{50} (ng/ml)	42.8 ± 9.2 (21.5%)	24.7–60.9
n_H	1.1 ± 0.2 (15.5%)	0.8–1.5
E_0 (μV)	20.4 ± 0.3 (1.5%)	19.8–21.0
Inter-occasion variability[b]		
$\omega^2_{E_{max},control}$ (IOV)	18.1 ± 5.8 (32.0%)	6.7–29.5
Residual error[c]		
$\sigma^2_{PD,control}$	12.5 ± 1.9 (15.1%)	8.8–16.2

[a] Parameter estimates ± SE. Between brackets coefficient of variation in %.
[b] Analysis of inter-individual (IIV) and inter-occasion variability (IOV) showed that the inter-occasion variability in maximal EEG-effect of midazolam (E_{max}) was significantly different from zero, indicating either random variability between occasions, or an effect of repeated administration of midazolam.
[c] An additive residual error accounts for any measurement/intra-individual error.

account inter-individual differences in midazolam EEG-effect, E_{max} of the second PK-PD experiment (at either day 4, or 14) was estimated as a fraction of the individual *post hoc* estimate of E_{max} of the first PK-PD experiment. This showed that there was a significant and permanent decrease of E_{max} after induction of status epilepticus. Specifically, at

Table 7.5: Mean values of E_{max} for the different treatment groups.

Treatment group	E_{max} (μV)[ab] 1st experiment	E_{max} (μV)[ab] 2nd experiment	day 2nd experiment
kainic acid-treated	10.4 ± 3.2	4.7 ± 2.2[c]	4
kainic acid-treated	11.2 ± 3.3	3.3 ± 5.2[d]	14
control	9.2 ± 3.1	9.4 ± 4.9[e]	4
control	12.4 ± 5.3	9.4 ± 6.1[e]	14

[a] Mean of *post hoc* estimates ± SD.
[b] Both inter-individual (IIV) and inter-occasion variability (IOV) for maximal EEG-effect of midazolam (E_{max}) were significantly greater than zero, resulting in individual *post hoc* estimates of E_{max} for each individual animal, and for each occasion (1st (before status epilepticus) or 2nd (afer status epilepticus)).
[c] Significantly different from corresponding 1st experiment (p = 0.015).
[d] Significantly different from corresponding 1st experiment (p = 0.0018).
[e] The differences between the 1st and 2nd experiment were not significant for the control groups ($p > 0.05$).

Table 7.6: Population estimates of PD-parameters and variabilities of experiments after status epilepticus.

Parameter	Value[a]		95% confidence interval
Structural parameters			
α^{b}	0.21 ± 0.088	(41.6%)	0.039–0.38
β^{cd}	0.037 ± 0.0032	(8.6%)	0.031–0.043
E_o (μV)^{d}	22.7 ± 1.25	(5.5%)	20.3–25.2
Inter-occasion variability[e]			
$\omega^2_{E_{max},KA}$	1.38 ± 0.81	(59.3%)	−0.22–2.96
$\omega^2_{E_{max},KA,ARG}{}^{d}$	0.69 ± 0.32	(46.6%)	0.060–1.32
$\omega^2_{E_o,KA}{}^{d}$	21.0 ± 8.4	(20.3%)	2.89–6.71
Residual error[f]			
$\sigma^2_{PD,KA}{}^{d}$	4.8 ± 0.97	(20.3%)	2.89–6.71

[a] Parameter estimates ± SE. Between brackets coefficient of variation in %.
[b] α is a factor to describe the fractional difference between E_{max} of the two midazolam experiments (pre-status epilepticus and post-status epilepticus experiment).
[c] β is a parameter describing the relation between *ex vivo* binding of flumazenil (B_{FMZ}) and E_{max}.
[d] Parameter estimates of model in which relation with autoradiography (ARG) is included.
[e] Inter-occasion variability in maximal EEG-effect (E_{max}) and baseline (E_o) was significantly different from zero, indicating either random variability between occasions, or an effect of induction of status epilepticus.
[f] The additive residual error accounts for any measurement/intra-individual error.

day 4 E_{max} was reduced to 51.6 ± 35.6%, and at day 14 this value was further reduced to 25.8 ± 33.7% (mean ± SD of individual *post hoc* estimates). The difference between day 4 and 14 was not significant, because of large inter-individual differences.

7.3.4 Correlations between midazolam EEG-effect and ex vivo [^{3}H]flumazenil binding

The relationship between the reduction in E_{max} and $GABA_A$ receptor density is shown in figure 7.3. In this figure the individual estimates of the relative difference in E_{max} are plotted *versus* the *ex vivo* measured relative decrease in [^{3}H]flumazenil binding in the cortex (B_{FMZ}). Induction of status epilepticus had only a small effect on the expression of the $GABA_A$ receptor, as B_{FMZ} was reduced to 90.6 ± 7.0% of control at day 4 after status epilepticus, and to 72.0 ± 14.9% of control at day 14 after status epilepticus.

To study this relation in more detail, the whole range in reduction in E_{max} and $GABA_A$ receptor density was used. Therefore, the results obtained at day 4 and 14 after status epilepticus were analysed simultaneously. Exploring various mathematical functions showed that the relation between *ex vivo* binding and *in vivo* effect as determined by [^{3}H]flumazenil autoradiography were best described by an exponential function. Implementing this function in the model resulted in a considerable decrease in random

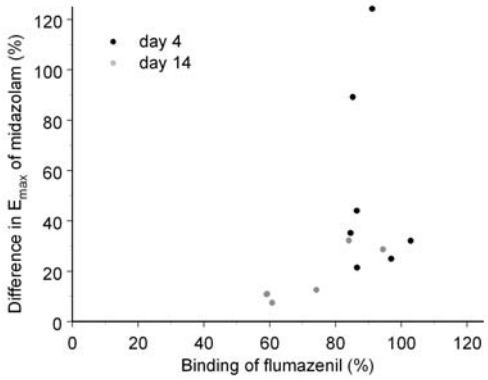

Figure 7.3: Correlation between binding of [^{3}H]flumazenil in the cortex (*ex vivo*), and *in vivo* maximal EEG-effect of midazolam. Binding in individual kainic acid-treated rats is shown as percentage of control animals, and maximal EEG-effect (E_{max}) of the second experiment (after status epilepticus) is shown as percentage of the corresponding value for E_{max} of the first experiment (difference in E_{max}).

variability of E_{max} across the population (from 1.38 to 0.69, table 7.6), indicating that part of this variability is indeed caused by individual differences in alterations of B_{max} of the $GABA_A$ receptor. The parameter estimates of the analysis of the experiments after induction of status epilepticus (kainic acid-treated animals) are presented in table 7.6. The individual *post hoc* estimates of E_{max} reported in table 7.5 are obtained from the model in which the *ex vivo* results with flumazenil are implemented. The results of this model are

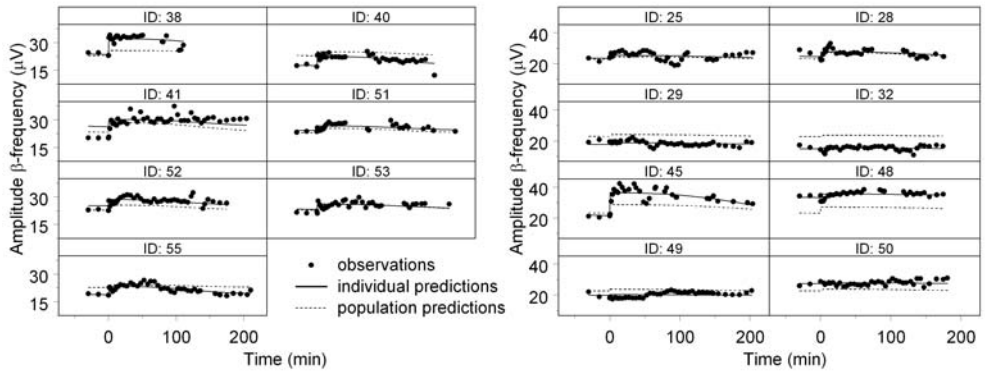

Figure 7.4: Individual fits of effect-time curves of PK-PD experiments with midazolam after induction of status epilepticus. Left panels: experiment performed at day 4 after status epilepticus; right panels: experiment performed at day 14 after status epilepticus. Symbols represent individual data, solid lines individual fits and dashed lines population fits. At t = 0 min 10 mg/kg midazolam was intravenously infused in 2 minutes.

also used for figure 7.4, in which the individual fits of the effect time curves of midazolam after induction of status epilepticus are shown.

7.4 Discussion

The main results of the present study are that (a) the maximal effect (E_{max}) of midazolam, as quantified by the amplitude of the β-frequency of the EEG, was decreased after induction of status epilepticus in animals, and (b) the observed decrease in midazolam-effect after kainic acid-treatment is partly related to a decline in [^{3}H]flumazenil binding in rat brains *ex vivo*.

The decrease in midazolam-effect after induction of status epilepticus might in principle be the result of alterations in either PK, or PD. Using a PK-PD approach, it was possible to exclude alterations in PK as a causal factor. In the present study, the PK of midazolam was described by a three-compartment model, whereas in previous studies the PK of midazolam was described by a two-compartment model.[25,22,26] On one hand, this may be related to the used rat strain: we used Sprague Dawley rats, whereas in previous studies Wistar rats are used.[25,22,26] More likely, however, it is because of the large amount of data available in the present study: the data of 50 rats were included, and two experiments per rat were performed. As the number of parameters which are identifiable in the model, is dependent on the amount of data available, this is a plausible explanation for the significant improvement of using a three-compartment model over a two-compartment model. The currently reported value of CL (24.6 ml/min) is comparable with previously reported values of 16.8 ml/min,[22] 22.7 ml/min,[26] 21.5 ml/min,[25] or 20.8 ml/min.[25] Also the value of $V_{d,ss}$ (total volume of distribution, 841 ml) is in the same range as the in literature reported values of 517 ml,[22] 547 ml,[26] 595 ml,[25] or 468 ml.[25]

An important issue to consider is whether status epilepticus altered the transport of midazolam across the blood-brain barrier, because upregulation of multidrug-resistance transporters, as reported in both human epileptogenic brain tissue and experimental models,[27,28] could lower the brain concentration of midazolam and explain a decrease in midazolam EEG-effect. The design of this study does not allow conclusions in this respect. However, in contrast to many antiepileptic drugs, midazolam is no substrate for multidrug transporters, whereas it has a rather high *in vitro* passive permeability, which is a good characteristic for delivery to the CNS.[29] Second, the sedative effect of midazolam immediately after administration was not altered by status epilepticus (personal observation), indicating that midazolam readily entered the brain.

The decrease in E_{max} could in fact be due to tolerance development as a result of the repeated administration of midazolam, rather than a result of status epilepticus.[30,31] To check this possibility, control groups, receiving saline instead of kainic acid, were included in the experimental setup. It was found that the effect of midazolam at the second experiment for these groups was not significantly different from their corresponding first experiments, implying that no significant tolerance had developed after the first administration of midazolam.

Although the between-day differences in midazolam effect of the kainic acid-treated groups were not statistically significant in this study, the data nevertheless suggest a progressive decrease in E_{max} of midazolam from day 4 to 14 after induction of status epilepticus. In fact, we believe that the absence of an effect of induction of status epilepticus in a few animals masked a true progressive decrease from day 4 to 14 after induction of status epilepticus. These inter-individual differences in midazolam-effect after induction of status epilepticus were also observed in the *ex vivo* studies with [^{3}H]labelled flumazenil, which showed that the observed reduction in EEG-effect of midazolam is parallelled by an impairment of maximal binding (B_{FMZ}). This implicates that a decrease in maximal binding to the $GABA_A$ receptor might be responsible for the observed decrease in efficacy of midazolam.

The underlying mechanism, causing the observed decrease in flumazenil-binding cannot be explained based on this experimental setting. Different alterations in GABAergic inhibition as result of epilepsy have been reported in literature. For instance, Goodkin *et al* showed that status epilepticus causes a rapid internalisation of $GABA_A$ receptors, resulting in a decreased receptor density.[32] However, this does not explain the discrepancy between the moderate reduction in flumazenil binding, and the more pronounced decrease in midazolam effect. Several explanations are possible for this observation. First, it might be that the populations of $GABA_A$ receptors reflected by flumazenil binding, and those giving rise to an increase in β-activity in the cortical EEG upon midazolam administration, do not completely overlap. Thus, there might be a selective reduction in a subpopulation of $GABA_A$ receptors that is responsible for the reduced efficacy of midazolam, whereas other receptors are unaffected, which would be reflected by a moderate reduction in flumazenil binding. The observation that the sedating effect of midazolam is not decreased, even in rats that had almost no EEG-effect of midazolam after induction of status epilepticus (personal observation), is consistent with this interpretation.

Furthermore, several groups have explored changes in subunit composition of the $GABA_A$ receptor as result of epilepsy. A decrease in α_2, α_3, α_5, β_1, β_3, γ_2, and δ subunits in the hippocampus, and an increase in α_1, α_4, and β_2 subunits was reported immediately after kainic acid-induced status epilepticus.[33,34] The rapid decrease in midazolam efficacy, as observed in the present study may be due to the subunit exchanges. For example, a loss or replacement of the γ_2 subunit would render the receptor insensitive to benzodiazepines. Furthermore, benzodiazepine sensitivity is conferred by the presence of an α_1, α_2, α_3, or α_5 subunit, thus replacement of these subunits for α_4 would result in a loss of midazolam effectivity,[35] whereas flumazenil binding is far less sensitive for subunit changes. Thus, the $GABA_A$ receptor might be altered, resulting in decreased affinity for midazolam, whereas the affinity for flumazenil is less affected.

7.5 Conclusion

In summary, induction of status epilepticus by repeated *ip* injections of kainic acid results in a decreased efficacy of midazolam, as reflected in a decrease in E_{max} after status epilepticus, which tends to be progressive. This decrease in effect runs in parallel with a decreased *ex vivo* binding of [^{3}H]flumazenil, indicating that the decline of midazolam effect is, at least partly, due to a reduced binding to the $GABA_A$ receptor.

Acknowledgements

The authors wish to thank K.B. Postel-Westra and Y. Tagawa for help with the surgeries and experiments, J.H.G. van Woezik for performing autoradiography experiments, and M.C.M. Blom-Roosemalen for help with the HPLC analysis of the blood samples.

This project was sponsored by the "Christelijke Vereniging voor de Verpleging van Lijders aan Epilepsie" and the National Epilepsy Fund – "The Power of the Small", project number 02-06.

References

1. W. Löscher. Current status and future directions in the pharmacotherapy of epilepsy. *Trends Pharmacol Sci*, 23(3):113–8., 2002.
2. G. Regesta and P. Tanganelli. Clinical aspects and biological bases of drug-resistant epilepsies. *Epilepsy Res*, 34(2-3):109–22, 1999.
3. W. Löscher. How to explain multidrug resistance in epilepsy? *Epilepsy Curr*, 5(3):107–12, 2005.
4. J. M. Fritschy, T. Kiener, V. Bouilleret, and F. Loup. Gabaergic neurons and gaba(a)-receptors in temporal lobe epilepsy. *Neurochem Int*, 34(5):435–45, 1999.
5. S. Remy and H. Beck. Molecular and cellular mechanisms of pharmacoresistance in epilepsy. *Brain*, 129(Pt 1):18–35, 2006.
6. H. F. Bradford. Glutamate, gaba and epilepsy. *Prog Neurobiol*, 47(6):477–511., 1995.
7. K. S. Hand, V. H. Baird, W. Van Paesschen, M. J. Koepp, T. Revesz, M. Thom, W. F. Harkness, J. S. Duncan, and N. G. Bowery. Central benzodiazepine receptor autoradiography in hippocampal sclerosis. *Br J Pharmacol*, 122(2):358–64, 1997.
8. S. Lamusuo, A. Pitkänen, L. Jutila, A. Ylinen, K. Partanen, R. Kalviainen, H. M. Ruottinen, V. Oikonen, K. Nagren, P. Lehikoinen, M. Vapalahti, P. Vainio, and J. O. Rinne. [11c]flumazenil binding in the medial temporal lobe in patients with temporal lobe epilepsy: correlation with hippocampal mr volumetry, t2 relaxometry, and neuropathology. *Neurology*, 54(12):2252–60., 2000.
9. L. K. Friedman, D. E. Pellegrini-Giampietro, E. F. Sperber, M. V. Bennett, S. L. Moshe, and R. S. Zukin. Kainate-induced status epilepticus alters glutamate and gabaa receptor gene expression in adult rat hippocampus: an in situ hybridization study. *J Neurosci*, 14(5 Pt 1):2697–707., 1994.
10. L. Rocha and R. Ondarza-Rovira. Characterization of benzodiazepine receptor binding following kainic acid administration: an autoradiography study in rats. *Neurosci Lett*, 262(3):211–4, 1999.

11. A. R. Brooks-Kayal, M. D. Shumate, H. Jin, T. Y. Rikhter, and D. A. Coulter. Selective changes in single cell gaba(a) receptor subunit expression and function in temporal lobe epilepsy. *Nat Med*, 4(10):1166–72, 1998.

12. D. M. Jones, N. Esmaeil, S. Maren, and R. L. Macdonald. Characterization of pharmacoresistance to benzodiazepines in the rat li-pilocarpine model of status epilepticus. *Epilepsy Res*, 50(3):301–12, 2002.

13. C. Brandt, H. A. Volk, and W. Löscher. Striking differences in individual anticonvulsant response to phenobarbital in rats with spontaneous seizures after status epilepticus. *Epilepsia*, 45(12):1488–97, 2004.

14. J. Dingemanse, M. Danhof, and D. D. Breimer. Pharmacokinetic-pharmacodynamic modeling of cns drug effects: an overview. *Pharmacol Ther*, 38(1):1–52, 1988.

15. J. W. Mandema, L. N. Sansom, M. C. Dios-Vieitez, M. Hollander-Jansen, and M. Danhof. Pharmacokinetic-pharmacodynamic modeling of the electroencephalographic effects of benzodiazepines. correlation with receptor binding and anticonvulsant activity. *J Pharmacol Exp Ther*, 257(1):472–8, 1991.

16. J. W. Mandema and M. Danhof. Electroencephalogram effect measures and relationships between pharmacokinetics and pharmacodynamics of centrally acting drugs. *Clin Pharmacokinet*, 23(3):191–215, 1992.

17. B. L. Sheiner and T. H. Grasela. An introduction to mixed effect modeling: Concepts, definitions, and justification. *J Pharmacokinet Biopharm*, 19(3):11S–24S, 1991.

18. P. O. Maitre, M. Buhrer, D. Thomson, and D. R. Stanski. A three-step approach combining bayesian regression and nonmem population analysis: application to midazolam. *J Pharmacokinet Biopharm*, 19(4):377–84, 1991.

19. J. W. Mandema, D. Verotta, and L. B. Sheiner. Building population pharmacokinetic–pharmacodynamic models. i. models for covariate effects. *J Pharmacokinet Biopharm*, 20(5):511–28, 1992.

20. J. L. Hellier, P. R. Patrylo, P. S. Buckmaster, and F. E. Dudek. Recurrent spontaneous motor seizures after repeated low-dose systemic treatment with kainate: assessment of a rat model of temporal lobe epilepsy. *Epilepsy Res*, 31(1):73–84., 1998.

21. J. W. Mandema, E. Tukker, and M. Danhof. Pharmacokinetic-pharmacodynamic modelling of the eeg effects of midazolam in individual rats: influence of rate and route of administration. *Br J Pharmacol*, 102(3):663–8, 1991.

22. S. A. Visser, F. L. Wolters, J. M. Gubbens-Stibbe, E. Tukker, P. H. Van Der Graaf, L. A. Peletier, and M. Danhof. Mechanism-based pharmacokinetic/pharmacodynamic modeling of the electroencephalogram effects of gabaa receptor modulators: in vitro-in vivo correlations. *J Pharmacol Exp Ther*, 304(1):88–101., 2003.

23. SL Beal and LB Sheiner. *NONMEM users guides (I-VIII)*. Globomax, Hanover, Maryland, 1989.

24. N. H. Holford and L. B. Sheiner. Understanding the dose-effect relationship: clinical application of pharmacokinetic-pharmacodynamic models. *Clin Pharmacokinet*, 6(6):429–53, 1981.

25. A. Cleton, R. A. Voskuyl, and M. Danhof. Adaptive changes in the pharmacodynamics of midazolam in different experimental models of epilepsy: kindling, cortical stimulation and genetic absence epilepsy. *Br J Pharmacol*, 125(4):615–20., 1998.

26. L. Aarons, J. W. Mandema, and M. Danhof. A population analysis of the pharmacokinetics and pharmacodynamics of midazolam in the rat. *J Pharmacokinet Biopharm*, 19(5):485–96, 1991.

27. E. Aronica, J. A. Gorter, M. Ramkema, S. Redeker, F. Ozbas-Gerceker, E. A. van Vliet, G. L. Scheffer, R. J. Scheper, P. van der Valk, J. C. Baayen, and D. Troost. Expression and cellular distribution of multidrug resistance-related proteins in the hippocampus of patients with mesial temporal lobe epilepsy. *Epilepsia*, 45(5):441–51, 2004.

28. E. A. van Vliet, S. Redeker, E. Aronica, P. M. Edelbroek, and J. A. Gorter. Expression of multidrug transporters mrp1, mrp2, and bcrp shortly after status epilepticus, during the latent period, and in chronic epileptic rats. *Epilepsia*, 46(10):1569–80, 2005.

29. K. M. Mahar Doan, J. E. Humphreys, L. O. Webster, S. A. Wring, L. J. Shampine, C. J. Serabjit-Singh, K. K. Adkison, and J. W. Polli. Passive permeability and p-glycoprotein-mediated efflux differentiate central nervous system (cns) and non-cns marketed drugs. *J Pharmacol Exp Ther*, 303(3):1029–37, 2002.

30. A. Cleton, J. Odman, P. H. Van der Graaf, W. Ghijsen, R. Voskuyl, and M. Danhof. Mechanism-based modeling of functional adaptation upon chronic treatment with midazolam. *Pharm Res*, 17(3):321–7., 2000.

31. M. A. Hutchinson, P. F. Smith, and C. L. Darlington. The behavioural and neuronal effects of the chronic administration of benzodiazepine anxiolytic and hypnotic drugs. *Prog Neurobiol*, 49(1):73–97., 1996.

32. H. P. Goodkin, J. L. Yeh, and J. Kapur. Status epilepticus increases the intracellular accumulation of gabaa receptors. *J Neurosci*, 25(23):5511–20, 2005.

33. G. Sperk, C. Schwarzer, K. Tsunashima, and S. Kandlhofer. Expression of gaba(a) receptor subunits in the hippocampus of the rat after kainic acid-induced seizures. *Epilepsy Res*, 32(1-2):129–39, 1998.

34. L. E. Nelson, T. Z. Guo, J. Lu, C. B. Saper, N. P. Franks, and M. Maze. The sedative component of anesthesia is mediated by gaba(a) receptors in an endogenous sleep pathway. *Nat Neurosci*, 5(10):979–84, 2002.

35. D. M. Jones-Davis and R. L. Macdonald. Gaba(a) receptor function and pharmacology in epilepsy and status epilepticus. *Curr Opin Pharmacol*, 3(1):12–8., 2003.