Chapter VII

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

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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 [3H]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 [3H]flumazenil showed that the observed reductions in E_max were paralleled 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$4

There is much evidence for alterations in GABA$_A$ receptors in mTLE, resulting in decreased GABAergic effects.$^5$ 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 $[^1]$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$10 Furthermore, rapid and extensive changes in subunit expression have been reported, paralleled by changes in in vitro pharmacology.$^{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 beta-frequency band of the EEG was used as a measure for the facilitation of GABA\(_A\) receptor mediated inhibition. The beta-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 beta-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 beta-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 (Hynpnom, Janssen Pharmaceutica, Tilburg, The Netherlands) and 18 mg/kg sodiumpentobarbital (Nembutal, Ceva Sante Animale, Maassluis, The Netherlands). Both anesthetics 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 sagittalis. 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. |
|--------------------------|----|----|----|----|
| Treatment group          | –2 | 4  | 14 | n  |
| kainic acid-treated      | x  | X  |    | 7  |
| kainic acid-treated*     | x  |    | X  | 8  |
| control                  | x  | x  |    | 5  |
| control*                | x  |    | x  | 6  |

*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".
*Animals of the kainic acid-treated groups were treated with kainic acid to induce status epilepticus.
*The control groups were treated with saline instead of kainic acid.
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.\textsuperscript{21} with the modifications described by Visser et al.\textsuperscript{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\textsubscript{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.\textsuperscript{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.\textsuperscript{17} By taking into account IIV and IOV, individual post hoc estimates of the structural parameters are obtained.\textsuperscript{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,\textsuperscript{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:\textsuperscript{24}

\[
E = E_0 + \frac{E_{max} \cdot C^{nH}}{EC_{50}^{nH} + C^{nH}}
\]

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 \(nH\) 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:

\[
E_{max,exp2} = \alpha \cdot E_{max,exp1}
\]

in which \(E_{max,exp1}\) represents the individual post hoc estimate of the \(E_{max}\) of the first (pre-status epilepticus) midazolam-experiment, and \(\alpha\) 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 \textit{ex vivo} binding of \(^{3H}\)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 \textit{ex vivo} binding of \(^{3H}\)flumazenil:

\[
E_{max,exp2} = e^{\beta \cdot BF_{MZ} \cdot \frac{E_{max,exp1}}{100}}
\]
in which $R_{FMZ}$ represents the percentage of binding of $[^3H]$flumazenil as measured \textit{ex vivo} (see “\textit{Ex vivo} binding studies with $[^3H]$flumazenil”). Also in this case the remaining IIV in $E_{max,exp}$ was described by an additive variance model.

7.2.8 \textit{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 \textit{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 \textbf{Results}  
7.3.1 \textit{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

\textbf{Figure 7.2:} Typical examples of midazolam plasma-concentration, and EEG-effect \textit{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.

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

$^a$ 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 \( V \) for the different treatment groups.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>( CL ) (ml/min)(^{a,b}) 1(^{st}) experiment</th>
<th>( CL ) (ml/min)(^{a,b}) 2(^{nd}) experiment</th>
<th>( V ) (ml)(^{c}) day</th>
<th>( V ) (ml)(^{c}) 2(^{nd}) experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>kainic acid-treated</td>
<td>30.9 ± 6.4</td>
<td>27.9 ± 3.3</td>
<td>529 ± 71.7</td>
<td>4</td>
</tr>
<tr>
<td>kainic acid-treated</td>
<td>30.8 ± 2.6</td>
<td>24.8 ± 5.4</td>
<td>603 ± 95.9</td>
<td>14</td>
</tr>
<tr>
<td>control</td>
<td>29.5 ± 4.7</td>
<td>31.2 ± 6.2</td>
<td>533 ± 44.8</td>
<td>4</td>
</tr>
<tr>
<td>control</td>
<td>27.3 ± 0.5</td>
<td>26.2 ± 2.3</td>
<td>504 ± 130</td>
<td>14</td>
</tr>
</tbody>
</table>

\(^a\)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 (1\(^{st}\) (before status epilepticus) or 2\(^{nd}\) (after status epilepticus)). There were no significant differences between experimental groups or occasions (1\(^{st}\) versus 2\(^{nd}\) experiment, \( p > 0.05 \)).
\(^c\)For \( V \), only IIV was significantly different from zero, thus individual post hoc estimates of \( V \) 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.

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</tr>
<tr>
<td>$E_{max}$ ($\mu$V)</td>
<td>10.4 ± 0.6 (5.8%)</td>
<td>9.2–11.6</td>
</tr>
<tr>
<td>$EC_{50}$ (ng/ml)</td>
<td>42.8 ± 9.2 (21.5%)</td>
<td>24.7–60.9</td>
</tr>
<tr>
<td>$n_H$</td>
<td>1.1 ± 0.2 (15.5%)</td>
<td>0.8–1.5</td>
</tr>
<tr>
<td>$E_0$ ($\mu$V)</td>
<td>20.4 ± 0.3 (1.5%)</td>
<td>19.8–21.0</td>
</tr>
<tr>
<td><strong>Inter-occasion variability</strong>$^{b}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\omega^2_{max,control}$ (IOV)</td>
<td>18.1 ± 5.8 (32.0%)</td>
<td>6.7–29.5</td>
</tr>
<tr>
<td><strong>Residual error</strong>$^{c}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\sigma^2_{PD,control}$</td>
<td>12.5 ± 1.9 (15.1%)</td>
<td>8.8–16.2</td>
</tr>
</tbody>
</table>

$^{a}$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.

To account for 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.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>$E_{max}$ ($\mu$V)$^{ab}$</th>
<th>day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$1^{st}$ experiment</td>
<td>$2^{nd}$ experiment</td>
</tr>
<tr>
<td>kainic acid-treated</td>
<td>10.4 ± 3.2</td>
<td>4.7 ± 2.2$^{e}$</td>
</tr>
<tr>
<td>kainic acid-treated</td>
<td>12.2 ± 3.3</td>
<td>3.3 ± 5.2$^{d}$</td>
</tr>
<tr>
<td>control</td>
<td>9.2 ± 3.1</td>
<td>9.4 ± 4.9$^{e}$</td>
</tr>
<tr>
<td>control</td>
<td>12.4 ± 5.3</td>
<td>9.4 ± 6.1$^{e}$</td>
</tr>
</tbody>
</table>

$^{a}$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 ($1^{st}$ (before status epilepticus) or $2^{nd}$ (after status epilepticus)).

$^{c}$Significantly different from corresponding $1^{st}$ experiment ($p = 0.015$).

$^{d}$Significantly different from corresponding $1^{st}$ experiment ($p = 0.0018$).

$^{e}$The differences between the $1^{st}$ and $2^{nd}$ 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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structural parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha^b$</td>
<td>$0.21 \pm 0.088$ (41.6%)</td>
<td>$0.039–0.38$</td>
</tr>
<tr>
<td>$\beta^c,d$</td>
<td>$0.037 \pm 0.0032$ (8.6%)</td>
<td>$0.031–0.043$</td>
</tr>
<tr>
<td>$E_0$ (µV)$^d$</td>
<td>$22.7 \pm 1.25$ (5.5%)</td>
<td>$20.3–25.2$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Inter-occasion variability$^e$</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$\omega_{E_{max},K_A}^3$</td>
<td>$1.38 \pm 0.81$ (59.3%)</td>
<td>$-0.22–2.96$</td>
</tr>
<tr>
<td>$\omega_{E_{max},K_A,ARG}^d$</td>
<td>$0.69 \pm 0.32$ (46.6%)</td>
<td>$0.060–1.32$</td>
</tr>
<tr>
<td>$\omega_{E_0,K_A}^d$</td>
<td>$21.0 \pm 8.4$ (20.3%)</td>
<td>$2.89–6.71$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Residual error$^f$</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$\sigma_{PD,KA}^d$</td>
<td>$4.8 \pm 0.97$ (20.3%)</td>
<td>$2.89–6.71$</td>
</tr>
</tbody>
</table>

$^a$Parameter estimates ± SE. Between brackets coefficient of variation in %.
$^b$ $\alpha$ is a factor to describe the fractional difference between $E_{max}$ of the two midazolam experiments (pre-status epilepticus and post-status epilepticus experiment).
$^c$ $\beta$ is a parameter describing the relation between ex vivo binding of flumazenil (BFMZ) 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 ($E_0$) 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 $51.6 \pm 35.6\%$, and at day 14 this value was further reduced to $25.8 \pm 33.7\%$ (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 $[^3H]$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 $[^3H]$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 $90.6 \pm 7.0\%$ of control at day 4 after status epilepticus, and to $72.0 \pm 14.9\%$ 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 $[^3H]$flumazenil autoradiography were best described by an exponential function. Implementing this function in the model resulted in a considerable decrease in random
variability of $E_{\text{max}}$ 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 $B_{\text{max}}$ 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 $E_{\text{max}}$ 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 7.3: Correlation between binding of [3H]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 ($E_{\text{max}}$) of the second experiment (after status epilepticus) is shown as percentage of the corresponding value for $E_{\text{max}}$ of the first experiment (difference in $E_{\text{max}}$).](image)

![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.](image)
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 ($E_{\text{max}}$) of midazolam, as quantified by the amplitude of the $\beta$-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. 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. 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 ml/min, 21.5 ml/min, or 20.8 ml/min. 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, 547 ml, 595 ml, or 468 ml.

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, 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. 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 $E_{\text{max}}$ could in fact be due to tolerance development as a result of the repeated administration of midazolam, rather than a result of status epilepticus. 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 $E_{\text{max}}$ 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 $[^{3}\text{H}]$labelled flumazenil, which showed that the observed reduction in EEG-effect of midazolam is paralleled by an impairment of maximal binding ($B_{\text{FMZ}}$). 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. 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 $\beta$-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 $\alpha_2$, $\alpha_3$, $\alpha_5$, $\beta_1$, $\beta_3$, $\gamma_2$, and $\delta$ subunits in the hippocampus, and an increase in $\alpha_1$, $\alpha_4$, and $\beta_2$ subunits was reported immediately after kainic acid-induced status epilepticus. 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 $\gamma_2$ subunit would render the receptor insensitive to benzodiazepines. Furthermore, benzodiazepine sensitivity is conferred by the presence of an $\alpha_1$, $\alpha_2$, $\alpha_3$, or $\alpha_4$ subunit, thus replacement of these subunits for $\alpha_4$ would result in a loss of midazolam effectivity, 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 [3H]flumazenil, indicating that the decline of midazolam effect is, at least partly, due to a reduced binding to the GABA$_A$ receptor.

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References


