Pharmacoresistance in epilepsy : modelling and prediction of disease progression

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Chapter VIII

Modulation of GABAergic inhibition by tiagabine and alphaxalone is differentially affected in the kainate model of temporal lobe epilepsy

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Summary

Purpose Previously, we studied the efficacy of midazolam in rats at 14 days after status epilepticus, induced by kainic acid. Modulation of $GABA_A$ mediated inhibition, reflected by enhancement of EEG β-activity, was decreased by 73%. This reduction was partly associated with a moderately decreased $GABA_A$ receptor density. To examine whether the reduced sensitivity was primarily due to reduced receptor density or altered receptor properties the facilitation of $GABA_A$ mediated inhibition by a GABA uptake blocker and a neurosteroid was studied.
Methods Status epilepticus was induced systemic injection of kainic acid. Pharmacokinetic-pharmacodynamic (PK-PD) experiments were performed with tiagabine and alphaxalone 2 days before and 14 days after induction of status epilepticus. The concentration-EEG effect relationship of tiagabine was modelled with a sigmoid function. The PK-PD model for the biphasic EEG effect of alphaxalone consisted of two parts. Receptor activation was modelled with a hyperbolic function of the drug concentration producing a stimulus. Subsequently transduction of the stimulus into the EEG effect was modelled with a parabolic function.
Results The concentration-effect relation of tiagabine 14 days after status epilepticus was unaltered compared to control. Modelling the effect of alphaxalone indicated a small, but significant increase in efficacy of 6–14%.
Conclusions Since tiagabine only prolongs the presence of GABA in the synaptic cleft, these results indicate that GABA release and receptor activation essentially remain intact after status epilepticus. The increased efficacy of alphaxalone points to alterations in $GABA_A$ receptor properties, which differentially affect the sensitivity to benzodiazepines and neurosteroids. Apparently, the moderate reduction in receptor density plays only a minor role.

8.1 Introduction

Mesial temporal lobe epilepsy (mTLE) is characterised by neuronal loss, gliosis, synaptic reorganisation and alterations in ion channel and receptor properties in selected regions

of the temporal lobe.[1,2] Dysfunctioning of $GABA_A$ receptor mediated inhibition is one of the mechanisms that has been implicated in mTLE. Several alterations in GABAergic inhibition could underlie this dysfunction, including a decrease in GABA synthesis, GABA release, $GABA_A$ receptor density, alteration in receptor properties or events downstream from receptor activation.[3] For instance, studies in both epileptic patients and animal models have shown that the binding of the benzodiazepine antagonist flumazenil is decreased, suggesting a decrease in the number of $GABA_A$ receptors.[4–7] Furthermore, diazepam and phenobarbital, which are modulators of GABAergic inhibition, are very effective in terminating pilocarpine-induced status epilepticus in rats when injected only a few minutes after the first stage III motor seizure, but quickly loose their efficacy at later time-points,[8] indicating rapid loss or alteration of $GABA_A$ receptors. Alterations in receptor properties could be due to the complex changes in subunit expression that have been reported in many studies in humans and animal models[9–16] and that are likely to affect both phasic and tonic inhibition.

To examine the mechanisms that could underlie this loss in drug efficacy we have compared the efficacy of the allosteric modulator midazolam before and after kainic acid-induced status epilepticus in rats.[17] The effect of midazolam was quantified by measuring the total power of the β-frequency band of the EEG, which reflects drug-induced GABAergic modulation in a quantitative manner, as shown previously.[18,19] The intrinsic efficacy of midazolam appeared to be decreased after induction of status epilepticus, as the maximal EEG effect (E_{max}) was reduced by 49% at day 4, and even by 73% at day 14 compared to control. Several explanations are possible. *Ex vivo* autoradiography studies with [^{3}H]flumazenil in the brains of these rats showed moderately impaired binding to the $GABA_A$ receptor, indicating either lower receptor density, or an altered receptor which does not bind flumazenil and presumably also not midazolam.[17] Alternatively the reduced efficacy could be secondary to a decreased availability or release of GABA or to events downstream of receptor activation by GABA. Our study could not discriminate between these alternatives.

The present study was undertaken with different ligands to obtain further insight in the adaptation of $GABA_A$ mediated inhibition in response to status epilepticus. The first ligand studied, was tiagabine, which inhibits the reuptake of GABA, thus resulting in prolonged presence of released GABA in the vicinity of receptors. As the action of tiagabine depends primarily on the amount of released GABA, it is expected that tiagabine efficacy is more sensitive to changes in the release process than to changes in subunit composition, because GABA does not exhibit large variability in affinity for synaptic $GABA_A$ receptor subtypes.

The second ligand was the synthetic neurosteroid alphaxalone. Neurosteroids are powerful allosteric modulators of GABA activation at all receptor subtypes, but preferentially at receptors containing δ subunits.[20–22] Such receptors are prominently present in the dentate gyrus and cerebellum, occur extrasynaptically and are important for tonic inhibition. Complex changes in expression of δ subunits have been observed

after induction of status epilepticus by pilocarpine.[9,23,13,16] Thus, alphaxalone can be used to assess changes in receptor subtypes that are insensitive to midazolam.

Both tiagabine and alphaxalone increase the amplitude of the β-frequency band of the EEG, reflecting enhancement of $GABA_A$ mediated inhibition.[24,25] Therefore, the influence of induction of status epilepticus on the efficacy of tiagabine and alphaxalone could be investigated with exactly the same study design as in our previous study with midazolam. Specifically, in individual rats the efficacy of the two ligands was tested in the post-status epilepticus animal model, in which status epilepticus is induced by repeated injections of kainic acid.[26] The first experiment was performed before induction of status epilepticus, and the second at day 14 after status epilepticus-induction. This second time point was chosen, because the study with midazolam showed the strongest reduction of maximal effect at post-status epilepticus day 14.[17]

To characterise the influence of status epilepticus, the potency and efficacy of tiagabine and alphaxalone were derived from the concentration-effect relationships. These relationships were determined using a population pharmacokinetic-pharmacodynamic (PK-PD) modelling approach. With this method the structural relation between the measured concentrations and concomittant EEG effects is quantitatively described, whilst taking into account inter-individual differences.[27] This approach has two key features. Firstly, for each ligand the control and kainic acid-treated animals are initially treated as one population and the average value for all parameter is estimated. Secondly, the variability in each parameter estimate is characterised and by covariate analysis it is examined whether the variability is caused by one or more of the study variables, such as status epilepticus or repeated drug application.[28–30] This is a more powerful statistical method than simple comparison of different groups[31] and therefore requires less animals. The data analysis was based on PK-PD models that were previously developed for tiagabine and alpahxalone.[24,25,32,33]

8.2 Methods

8.2.1 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 the Declaration of Helsinki and Dutch laws on animal experimentation. All experiments were approved by the Ethics Committee for Animal Experiments of the Leiden University.

8.2.2 Experimental setup

In total 20 rats were used, which were divided into 10 rats for the experiments with tiagabine, and 10 rats for the alphaxalone experiments. These groups were subdivided into control-treated animals (n = 4), and animals subjected to kainic acid-treatment (n = 6). A schematic representation of the experimental setup is shown in figure 8.1.

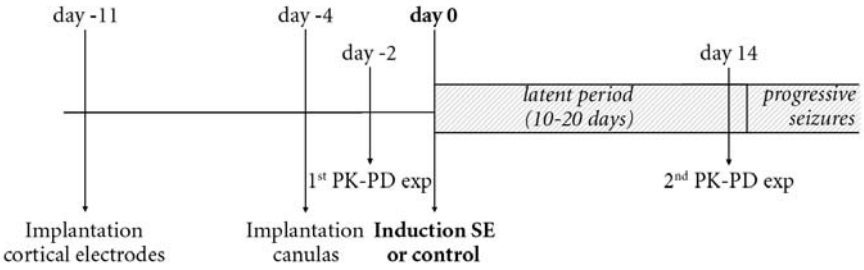

Figure 8.1: Schematic overview of experimental design. Two PK-PD experiments with either alphaxalone or tiagabine were performed in each animal: the first experiment before and the second experiment at day 14 after status epilepticus (SE) or control. Status epilepticus was induced by repetitive intraperitoneal injections with kainic acid. Control animals received saline injections.

For EEG recording cortical electrodes 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 midline. 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 PK-PD experiments with either tiagabine, or alphaxalone were performed in each animal: the first experiment was performed 2 days before induction of status epilepticus or injection of the vehicle, and the second experiment at day 14 after status epilepticus or injection of the vehicle.

Two days before the first PK-PD experiment with tiagabine or alphaxalone, 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).

8.2.3 Induction of status epilepticus

Status epilepticus was induced by repeated intraperitoneal injections with kainic acid according to the method described by Hellier *et al.*[26] Briefly, kainic acid, dissolved in saline, was injected intraperitoneally once per hour: the first dose was 10 mg/kg, all other injections were 5 mg/kg. Injections were given until class IV/V motor seizures (according to Racine's scale) occurred, or a total amount of 30 mg/kg kainic acid was administered. Typically, 3 injections with kainic acid were needed. The rats from the control groups received intraperitoneal injections with saline.

8.2.4 Drugs and dosages

Tiagabine was kindly donated by Sanofi-Synthelabo (Montpellier, France) Alphaxalone (5α-pregnan-3α-ol-11,20-dione) was purchased from Sigma Alldrich BV (Zwijndrecht, The Netherlands). Tiagabine was dissolved in water, and administered in a dose of 10 mg/kg in 10 min. Alphaxalone was dissolved in DMSO (Baker, Deventer, The Netherlands), and administered in a dose of 10 mg/kg in 5 min.

8.2.5 PK-PD experiment with tiagabine or alphaxalone

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 (maximally 210 min). 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 tiagabine infusion the following intervals were used: 10 intervals of 1 minute, 70 intervals of 2 minutes, and 3 intervals of 10 minutes, subsequently. From start of alphaxalone infusion the following intervals were used: 10 intervals of 0.5 minute, 70 intervals of 1 minute, and 7 intervals of 10 minutes, subsequently.

After recording of EEG baseline for 30 min, either tiagabine or alphaxalone was administered as a zero-order intravenous infusion to the conscious and freely moving rat using an infusion pump (Harvard Apparatus, South Natick, MA, USA). Serial arterial blood samples of 50–200 μl (larger samples at the end of the experiment) were taken at t = 0, 5, 10, 15, 20, 30, 40, 60, 90, 120, 150, and 180 min after start of infusion of tiagabine, or t = 0, 2.5, 5, 7.5, 10, 15, 20, 30, 60, 90, 120, 150, 180 min after start of infusion of alphaxalone. Total volume of blood samples was kept equal to 1.1 ml during each experiment. The blood samples were heparinised and centrifuged at 5000 rpm for 15 min for plasma collection. The plasma samples were stored at −20 °C until HPLC analysis.

8.2.6 Drug analysis in plasma

The plasma concentrations of tiagabine were determined by HPLC using UV detection. Desmethyltiagabine (DMT) was used as internal standard. Sample preparation was performed by elution with 3 times 0.5 ml ethanol through Oasis-HLB solid phase extraction columns (30 mg; Waters, Milford, MA). In the higher concentration range of tiagabine (>500 ng/ml) the eluate was simply diluted with 0.3 ml water and the mixture was directly injected. In the lower concentration range the eluate was evaporated at 40 °C (Vortex Evaporator, type 3-2201, Buchler) and the residue was dissolved in 300 μl acetonitrile:water (5:95 (v/v)). The injection volume was 200 μl.

Separation was performed at room temperature on an Inertsil 3ODS3 column (150 mm, 4.6 mm, Varian, Houten, The Netherlands) and UV-detection at 300 nm. The mobile phase consisted of methanol, acetonitrile, and 20 mM phosphate buffer (pH 7.0), in a ratio of 26:26:48 (v/v/v). At a flow rate of 1.0 ml/min the retention times of tiagabine and DMT were 13.0 and

9.8 min, respectively. Concentrations of tiagabine were calculated using the ratio of the peak heights of tiagabine and DMT. The limit of quantification was 25 ng/ml tiagabine with 100 μl of plasma. A linear calibration curve could be obtained in the range of 0–6,000 ng/ml (R^2 = 0.999; n = 10). The recovery of tiagabine was 88% at a concentration of 3,000 ng/ml (n = 6). Within-day precision for tiagabine was 3.7% at 3,000 ng/ml (n = 10); the day-to-day precision was 4.8% at 1,500 ng/ml (n = 8).

Alphaxalone concentrations in plasma were analysed using a high-performance liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) method. The volumes of the samples were adjusted to 100 μl with blank plasma, and 10 μl 10,000 ng/ml pregnanolone (Sigma Alldrich, Japan) was added as internal standard. Subsequently the samples were deproteinised by precipitation with 150 μl acetonitrile (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After centrifugation, the supernatant was diluted with 200 μl 0.4% acetic acid solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). 2 μl of the processed samples were injected into a LC/MS/MS system.

The LC/MS/MS system consisted of a Shimadzu LC-10ADvp solvent pump (Shimadzu, Kyoto, Japan), a SIL-HTC automatic sample injector with cooling system, set at 10 °C (Shimadzu, Kyoto, Japan), a Shimadzu CTO-10ACvp column oven, set at 50 °C (Shimadzu, Kyoto, Japan), an Inertsil Ph-3 column (5 micron, 150 mm, 2.1 mm, of GL Sciences Inc., Tokyo, Japan), and an API3000 mass-spectrometer (AB/MDS Sciex Instrument, CA, USA). Ionisation of the compounds was performed using turbo ion spray (turbo probe temperature: 425 °C) in positive-ion mode. The ionspray voltage was set at 5.2 kV, with collision energies of 21 and 17 V for alphaxalone and pregnanolone respectively. The mobile phase consisted of a mixture of 0.4% acetic acid solution, methanol and acetonitrile at a ratio of 35:55:10 with a flow rate of 0.2 ml/min. Run time was 8 minutes. Alphaxalone (m/z 333.2→297.2) and pregnanolone (internal standard, m/z 317.3→281.3) were detected using a selected reaction monitoring mode. Concentrations of alphaxalone were determined from the peak area ratios of alphaxalone versus internal standard. The limit of quantification was 25.0 ng/ml. Linear calibration curves were obtained in the range of 25–10,000 ng/ml. The intra-day assay coefficient of variation at 75.0, 1,000.0, and 8,000.0 ng/ml were 5.1, 5.0, and 8.7% (n = 5) respectively, and accuracy (relative error) were 7.9, 4.6, and 1.5% (n = 5) respectively. Data acquisition and data analysis was performed using Analyst (version 1.3, AB/MDS Sciex Instrument, CA, USA).

8.2.7 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.[27] Shortly, population analysis results in estimates of three types of parameters: 1) the structural model parameters, which are average values for the population of the parameters describing the dose-concentration-effect relationships, 2) inter-individual (IIV) and inter-occasion (IOV) variances and covariances of the structural model parameters, and 3) residual variance, which is associated with measurement/intraindividual error.[27] In the present study design an IIV significantly different from zero indicates differences due to the fact that two individual animals are never identical, whereas an IOV significantly different from zero can be due to the effect of status epilepticus, to repeated drug administration or to changes related to the lapse of time (e.g., aging, weight changes). By taking into account IIV and IOV, *post hoc* estimates of the structural parameters are obtained for each individual animal.[34] 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,[34] is used as measure of the precision of parameter estimation.

To analyse the PK-PD relationship of tiagabine and alphaxalone, a step-wise approach was applied. The first step consisted of the modelling of the PK of the ligands with the use of a two-compartment model for tiagabine, or a three-compartment model for alphaxalone, resulting in ligand-specific estimates of total body clearance (CL), intercompartmental clearances (Q_2, and Q_3), and volumes of distribution of the different compartments (V_1, V_2, and V_3). All 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 for both compounds.

Using individual *post hoc* estimates of the structural PK-parameters, concentrations at the PD-observation time points were simulated. To account for a delay in EEG-response compared to the plasma concentration profiles of both tiagabine and alphaxalone, an effect-compartment with a first-order rate constant k_{e0} was used,[35] resulting in the biophase concentration (C_e) versus time profile.

In the second step the relation between concentration and EEG-effect was described. As shown previously, the EEG-effect of tiagabine is best described by the sigmoidal E_{max}-equation:[24]

$$E = E_0 + \frac{E_{max} \cdot C_e^{n_H}}{EC_{50}^{n_H} + C_e^{n_H}} \tag{8.1}$$

in which the EEG-effect (E) is a function of the biophase concentration (C_e). The parameters E_0 (baseline EEG activity), E_{max} (maximal response to tiagabine), EC_{50} (concentration at half-maximal response), and n_H (Hill factor) were estimated.

The EEG-effect of alphaxalone was described with the mechanism-based PK-PD model described by Visser *et al.* This model is based on a separate characterisation of the receptor-activation process and the stimulus-response relationship.[32] The receptor-activation process is described by a hyperbolic function, in which the interaction with the receptor yields a stimulus S, reflecting the activation of the receptor:

$$S = \frac{e_{PD} \cdot C_e}{K_{PD} + C_e} \tag{8.2}$$

S is a function of the biophase concentration (C_e), e_{PD} is the *in vivo* efficacy, expressed as fraction of the maximally possible efficacy (1 being the value for the most effective ligand in a series of analogous compounds), and K_{PD} is the *in vivo* potency. Since the stimulus-response relationship has a biphasic shape (i.e., a maximum at low concentration followed by a decrease below baseline with increasing concentration) this relationship is described by a parabolic function:

$$E = E_0 - a \cdot \left((S^d)^2 - 2 \cdot b \cdot S^d \right) \tag{8.3}$$

in which E_0 represents the baseline EEG activity, a is a constant reflecting the slopes of the parabola, and $b^{1/d}$ is the stimulus for which the top of the parabola (i.e., the maximal increase in β-frequency of the EEG) is reached. The parameters k_{e0}, K_{PD}, and a were estimated. Because alphaxalone acts as a highly effective agonist, the value of e_{PD} in control animals was fixed at 1. To be able to estimate the value of the parameter b, the effect of alphaxalone at maximal stimulus should drop below baseline values, as shown by Visser *et al.* They also showed that the parameter d could only be estimated if a series of different neuroactive steroids was analysed simultaneously.

Since in the current investigation only alphaxalone was investigated, and its effect at maximal concentrations did not drop below baseline values (see figure 8.4, for an example), both b and d were fixed at the values reported by Visser *et al* (b = 0.44, $d = 3^{32}$).

All IIV and IOV in any parameter were described with an exponential variance model. An additive error model was used to characterise residual errors.

The effect of treatment with kainic acid on any parameter describing the concentration-effect relationship for both tiagabine and alphaxalone was studied by estimating a fractional difference between parameter values of the first (pre-status epilepticus) and the second (post-status epilepticus) experiment:

$$P_{exp2} = (1 + \alpha) \cdot P_{exp1} \tag{8.4}$$

in which P_{exp1} and P_{exp2} represents the estimate of the inspected parameter of the first (pre-status epilepticus), or second (post-status epilepticus) experiment respectively, and α the fractional difference between these parameter estimates. The value for α can be either positive, representing an increase in effect, or negative, representing a decrease.

8.2.8 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 1) whether the variability was significantly different from zero, 2) whether inclusion did not affect estimation of any other parameter, and 3) whether inclusion improved goodness of fit. Furthermore, in case of a significant

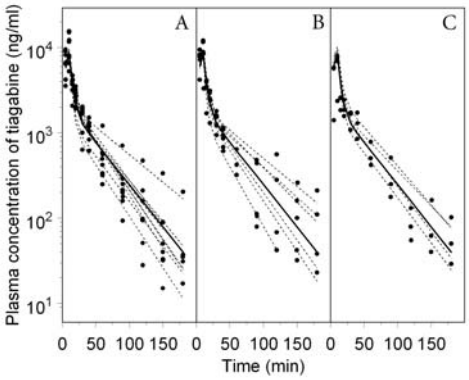

Figure 8.2: Individual fits of concentration-time curves of PK-PD experiments with tiagabine in controls or after induction of status epilepticus. Panel A: experiment performed before induction of status epilepticus or saline injection; panel B: experiment performed at day 14 after status epilepticus; panel C: experiment performed at day 14 after saline injection. Symbols represent individual data, solid lines represent population fit, and dashed lines represent fits of individual animals. At t = 0 min 10 mg/kg tiagabine was intravenously infused in 10 minutes.

effect of treatment with kainic acid on a parameter, the factor α will be significantly different from zero.

As stated above, if IIV was significantly different from zero for any parameter, this means that individuals of the population truely differ with respect to this parameter. Any IOV significantly different from zero indicates an effect of status epilepticus, of repeating drug administration, an effect associated with the lapse of time or a combination of these factors.

Statistical analysis was performed using paired *t*-test for comparison of *post hoc* estimates of first and second experiments. Statistical tests were performed using InStat version 3.0 for Windows (GraphPad, San Diego, USA).

8.3 Results

8.3.1 Pharmacokinetics of tiagabine and alphaxalone

The concentration-time profile of tiagabine was best described with a two-compartment model, whereas the concentration-time profile of alphaxalone was best described with a three-compartment model, based on goodness of fit criteria. In figures 8.2 and 8.3 the concentration-time profiles of tiagabine and alphaxalone with their fits are presented. For both compounds, the concentration-time data of all rats were analysed simultaneously, which resulted in the accurate and precise estimation of all model parameters, as presented in table 8.1. For tiagabine, significant inter-individual variability (IIV) was observed for both clearance (CL) and the volume of distribution of the peripheral compartment (V_2). In addition, CL appeared to differ between the first (pre-status epilepticus) and second (post-status epilepticus) PK-PD experiment. The clearance (CL) of alphaxalone was also different between the two experiments. To examine whether this

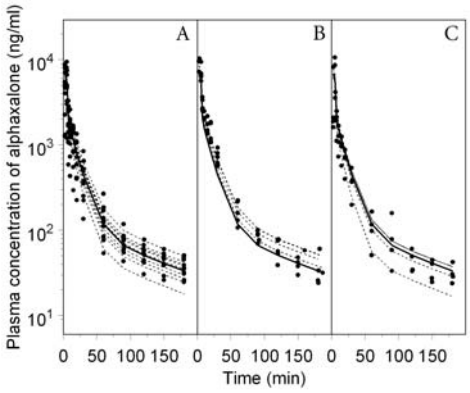

Figure 8.3: Individual fits of concentration-time curves of PK-PD experiments with alphaxalone in controls or after induction of status epilepticus or saline injection. Panel A: experiment performed before induction of status epilepticus or saline injection; panel B: experiment performed at day 14 after status epilepticus; panel C: experiment performed at day 14 after saline injection. Symbols represent individual data, solid lines represent population fit, and dashed lines represent fits of individual animals. At t = 0 min 10 mg/kg alphaxalone was intravenously infused in 5 minutes.

Table 8.1: Population estimates of PK-parameters for all ligands.

Parameter	Tiagabine[a]	Alphaxalone[a]
Structural parameters		
CL (ml/min)	16.3 ± 1.1 (6.9%)	41.0 ± 2.0 (2.0%)
Q_2 (ml/min)	13.5 ± 2.0 (15.0%)	9.0 ± 1.2 (12.7%)
Q_3 (ml/min)	NA[b]	47.5 ± 2.1 (4.4%)
V_1 (ml)	141 ± 11.8 (8.4%)	91.5 ± 4.6 (5.0%)
V_2 (ml)	291 ± 27.3 (9.4%)	1080 ± 315 (29.0%)
V_3 (ml)	NA[b]	326 ± 18.7 (5.7%)
Inter-individual & inter-occasion variability[c]		
IIV in CL	14.9%	NA[c]
IOV in CL	16.9%	17.2%
IOV in V_2	19.1%	NA[c]
Residual error[d]		
σ^2_{PK}	0.098 ± 0.015 (16.0%)	0.097 ± 0.017 (18.0%)

[a] Parameter estimates ± SE. Between brackets coefficient of variation in %.
[b] Not available.
[c] If significant inter-individual and inter-occasion variability was found, this is presented as percentage variation within the population, or between occasions.
[d] The residual error (σ^2), which accounts for any measurement/intra-individual error, is assumed to be proportional to the concentration of tiagabine or alphaxalone in blood.

observed difference was due to status epilepticus, repeating administration, or resulted from other changes between experiments, the individual *post hoc* parameter estimates for the first and the second PK-PD experiment were compared. This showed that the PK-parameters of both tiagabine and alphaxalone were neither affected by repeating administration nor by KA treatment.

8.3.2 Pharmacodynamics of tiagabine

In figure 8.4, typical examples of effect-time curves of tiagabine before and after induction of status epilepticus are shown. This figure shows that the effect of tiagabine at day 14 after status epilepticus is comparable to the control effect. The analysis of the tiagabine pharmacodynamics was performed in two steps. Firstly, all data of the first (pre-status epilepticus) PK-PD experiment with the GABA-reuptake inhibitor tiagabine were analysed using the sigmoidal E_{max}-model, including an effect-compartment to minimise the hysteresis (=delay) between plasma concentrations and EEG-effect. The results of this analysis are tabulated in table 8.2. It was not possible to estimate the Hill factor (n_H) with

Table 8.2: Population estimates of PD-parameters of tiagabine.

Parameter	First experiment[a]	All data[a b]
Structural parameters		
k_{e0} (1/min)	0.026 ± 0.0069 (26%)	0.031 ± 0.0078 (26%)
E_{max} (μV)	16.8 ± 2.4 (14%)	11.5 ± 1.4 (12%)
EC_{50} (ng/ml)	692 ± 134 (19%)	595 ± 90.6 (15%)
n_H	1.3 (fixed)	2.3 ± 0.35 (15%)
E_0 (μV)	21.1 ± 0.88 (4.2%)	23.0 ± 0.66 (2.9%)
Inter-individual variability[c]		
IIV in E_{max}	40.2%	54.5%
IIV in EC_{50}	NA[d]	61.4%
Residual error[e]		
σ^2_{PD}	14.6 ± 4.8 (33%)	14.8 ± 4.0 (27%)

[a] Parameter estimates ± SE. Between brackets coefficient of variation in %.
[b] Simultaneous analysis of all data.
[c] If significant inter-individual variability was found, this is presented as percentage variation within the population.
[d] Not available.
[e] The additive residual error (σ^2) accounts for any measurement/intra-individual error.

adequate precision. Numerical evaluation revealed that the value of n_H was likely to be about 1.3, thus n_H was fixed to this value.

Subsequently, the data from the second (post-status epilepticus) PK-PD experiment were added, and all data were analysed simultaneously, resulting in the parameter estimates as presented in table 8.2. All animals of the kainic acid-treated group underwent a status epilepticus of at least three hours upon treatment with kainic acid. On basis of the complete dataset, it was possible to estimate all parameters with adequate precision, including the Hill factor (n_H). This analysis shows two important findings. Firstly, since inter-occasion variability (IOV) was not different from zero for any parameter, there are no significant differences between the first (pre-status epilepticus) and second (post-status epilepticus) experiments. This indicates that neither status epilepticus nor repeated administration of tiagabine affects the pharmacodynamics of tiagabine. Consistent with this finding the fractional difference (α) between parameter values of the first (pre-status epilepticus) and the second (post-status epilepticus) experiment was not different from zero for any PD parameter of the tiagabine EEG effect.

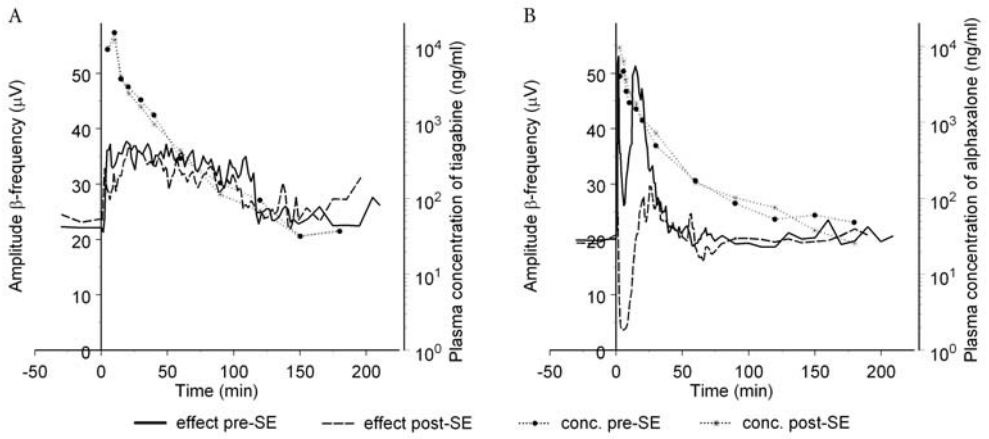

Figure 8.4: Typical example of plasma-concentration, and EEG-effect *versus* time curves of tiagabine (panel A) or alphaxalone (panel B) before and after induction of status epilepticus (SE) with kainic acid. At t = 0 min 10 mg/kg tiagabine or alphaxalone was intravenously infused in 10 minutes (tiagabine) or 5 minutes (alphaxalone).

8.3.3 Pharmacodynamics of alphaxalone

The EEG-effect of alphaxalone before induction of status epilepticus is characterised by a typical biphasic pattern, as shown in figure 8.4. Upon start of the infusion, the total amplitude of the β-frequency band (11.5–30 Hz) immediately increased, followed by a partial decrease below the the baseline value. After termination of the infusion when the plasma concentration started to decline, the effect increased again to the same height and then gradually returned to baseline. This biphasic pattern, which has also been observed in previous investigations in our lab and in studies with other anaesthetics,[36–38] was successfully described with the PK-PD model developed by Visser *et al.*[32] Simultaneous analysis of all data of the first (pre-status epilepticus) PK-PD experiment with alphaxalone resulted in precise estimates of all PD-parameters, including k_{e0} for hysteresis minimisation, which are shown in table 8.3.

After induction of status epilepticus, the EEG-effect of alphaxalone still shows a biphasic pattern, but the initial increase was smaller, whereas the subsequent decrease was more pronounced (figure 8.4). Analysis of all data of both the first (pre-status epilepticus) and second (post-status epilepticus) experiment simultaneously resulted in precise parameter estimates as shown in table 8.3. IOV for the parameters e_{PD} and a (or e_{PD} and b; see below) was significantly greater than zero As this effect could be completely attributed to the induction of status epilepticus, this implicates that repeated injections with alphaxalone did not affect the PK-PD of alphaxalone.

As shown in table 8.3, both the drug receptor interaction (as reflected in the parameter e_{PD}), and the stimulus-response relationship (as reflected in the parameters a or b) of alphaxalone were altered as result of treatment with kainic acid. In the stimulus-response

Table 8.3: Population estimates of PD-parameters of alphaxalone.

Parameter	First experiment[a]	All data[a b]
Structural parameters		
k_{eo} (1/min)	0.40 ± 0.042 (11%)	0.33 ± 0.019 (5.8%)
K_{PD} (ng/ml)	590 ± 90.5 (15%)	564 ± 46.2 (8.2%)
a	125 ± 7.6 (6.1%)	118 ± 8.1 (6.8%)
E_o (μV)	19.8 ± 0.68 (3.4%)	20.5 ± 0.70 (3.4%)
$\alpha_{KA,e_{PD}}$[c]	NA[d]	0.14 ± 0.020 (15%)
$\alpha_{KA,a}$	NA[d]	−0.36 ± 0.10 (29%)
$\alpha_{KA,e_{PD}}$[e]	NA[e]	0.056 ± 0.021 (37%)
$\alpha_{KA,b}$	NA[d]	−0.20 ± 0.064 (33%)
Inter-individual variability[f]		
IIV in a	20.7%	21.5%
Residual error[g]		
σ^2_{PD}	28.4 ± 4.9 (17%)	28.6 ± 2.7 (9.5%)

[a] Parameter estimates ± SE. Between brackets coefficient of variation in %.
[b] Simultaneous analysis of all data. Treatment with kainic acid appeared to affect either e_{PD} and a, or e_{PD} and b. No difference between these scenarios was found for the estimates of the other parameters.
[c] Difference in e_{PD} in case of alteration in a.
[d] Not available.
[e] Difference in e_{PD} in case of alteration in b.
[f] If significant inter-individual variability was found, this is presented as percentage variation within the population.
[g] The additive residual error (σ^2) accounts for any measurement/intra-individual error.

relationship, kainic acid-treatment affected either the value of parameter a ($\alpha_{KA,a} = -0.36$, indicating that a is decreased to 64% of control), or b ($\alpha_{KA,b} = -0.20$, indicating that b is decreased to 80% of control). On the basis of the current dataset it appeared impossible to discriminate between an alteration in a, or in b. The *in vivo* efficacy (e_{PD}) was significantly increased in both cases: to 114% (in case of alteration in a: $\alpha_{KA,ePD} = 0.14$) or 106% of control (in case of alteration in b: $\alpha_{KA,ePD} = 0.056$).

8.4 Discussion

This study has shown that status epilepticus induced by kainic acid administration does not diminish the efficacy of tiagabine and alphaxalone, as assessed by monitoring the change in β-activity in the EEG as a measure for facilitation of GABAergic inhibition. Rather, the efficacy of alphaxalone was slightly increased. These results are in contrast to

our previous study, which demonstrated a 73% reduction in efficacy of midazolam under identical conditions.[17] Together, this suggests that the properties of $GABA_A$ receptor mediated inhibition are altered by status epilepticus, but not impaired.

Alterations in pharmacokinetics in response to status epilepticus and the subsequent process of epileptogenesis could confound the assessment of pharmacological drug effect. Therefore, an integrated PK-PD approach was used to study the effects of tiagabine and alphaxalone. As shown in the present study, kainic acid-treatment did not affect the pharmacokinetics of either tiagabine or alphaxalone, indicating that all observed differences in pharmacodynamics are due to alterations in the concentration-effect relation.

No alteration was found in the pharmacodynamics of tiagabine, as measured by total power of the β-frequency band of the EEG, after induction of status epilepticus. This is in contrast to the results obtained in the amygdala kindling model, as Cleton *et al* reported a significant reduction in EC_{50}, implying that less tiagabine was needed to reach half-maximal effect, whereas the maximal effect (E_{max}) was not altered.[39] This leftward shift of the tiagabine concentration-EEG effect relationship was explained by an increased baseline GABA concentration, as revealed by intracerebral microdialysis. Since tiagabine administration in kindled animals resulted at every time-point in higher concentrations of GABA than in controls, the increase in baseline GABA level apparently added to or amplified the effect of blockade of the uptake carrier, thus lowering the EC_{50} of the EEG effect of tiagabine. We did not measure brain GABA concentrations in this study, but a study by Hasegawa showed that in the kainic acid-induced post-status epilepticus model, baseline GABA concentrations were not elevated.[40] It remains to be demonstrated what causes the difference between the kindling model and the post-status epilepticus model, but apart from that, the fact that the EEG-effect of tiagabine is unaltered suggests that GABAergic inhibition itself remains intact after status epilepticus.

From analysis of the alphaxalone data with the mechanism-based PK-PD model of Visser *et al*[32] two important conclusions can be drawn. First, the efficacy of alphaxalone, as reflected by the parameter e_{PD}, is increased after status epilepticus. This efficacy is the product of the intrinsic efficacy of a drug to initiate a stimulus from one receptor, ϵ, and the total number of receptors, R_t.[32] Thus, an increase in the efficacy of alphaxalone, suggests an increase in either the intrinsic efficacy, or the total number of receptors, or both. As the intrinsic efficacy is a strictly drug related parameter,[41] it is more likely that the induction of status epilepticus and the subsequent development of epilepsy changes the number of receptors. The second conclusion is that the change in the stimulus-response relationship, as reflected by the parameters a or b, means that the transduction process downstream of the receptor activation is altered. In other words, the results indicate that the neuronal circuits which generate the observed EEG-effects by enhancement of $GABA_A$ receptor mediated inhibition are functionally affected by epilepsy. At present, it remains to be demonstrated which of the two parameters (a, or b), characterising the transduction process, is altered by status epilepticus and what their actual physiological meaning

is. Nevertheless, it is clear that modulation of the $GABA_A$ receptors by alphaxalone is enhanced after status epilepticus, rather than impaired.

Taken together, the unaltered effect of tiagabine, the enhanced effect of alphaxalone, and the strongly reduced effect of midazolam at 14 days after status epilepticus suggest a selective alteration of the $GABA_A$ receptor after status epilepticus-induction, resulting in a receptor, which is more sensitive to modulation by alphaxalone, but less sensitive to midazolam modulation. The pharmacological and electrophysiological properties of the $GABA_A$ receptor are known to be dependent on the subunit composition of the receptor.[42] For instance, benzodiazepine sensitivity is conferred by the presence of an α_1, α_2, α_3, or α_5 subunit, whereas α_4 causes midazolam ineffectivity.[3] Furthermore, $GABA_A$ receptors without a γ_2 subunit are insensitive to benzodiazepines.[3] Neurosteroids, on the other hand, are specifically active on $GABA_A$ receptors with a δ subunit.[21] Therefore, one simple explanation for the observed differences between the changes in effectivity of midazolam and alphaxalone following induction of status epilepticus, could be an exchange of γ_2 subunits for δ subunits. Since the subunit composition of the $GABA_A$ receptor has relatively little influence on the activation of the receptor by GABA itself, it is likely that reduction of GABA reuptake by tiagabine would not be affected appreciably by exchange of receptor subunits, which is consistent with our results.

However, the results of several studies, in which the changes in subunit composition of the $GABA_A$ receptor as result of epileptogenesis were explored, suggest that the mechanism is likely to be more complicated. For instance, immediately after induction of status epilepticus with kainic acid or pilocarpine in rats, a decrease in α_2, α_3, α_5, γ_2 and δ subunits was observed in the dentate gyrus of the hippocampus, whereas α_1 and α_4 subunits were increased in this same area.[16, 23, 13] On the other hand, during the latent period, α_3, α_4 and δ subunits were shown to be increased in single dentate granule cells after induction of status epilepticus with pilocarpine, whereas the amount of γ_2 subunits was unaltered.[9, 13] Thus, various alterations in subunits after induction of status epilepticus are reported, but these alterations varied among different cell types or brain areas. This indicates that just a simple replacement of one subunit into another, for instance γ_2 into δ, resulting in a receptor which is more sensitive to alphaxalone than to midazolam, cannot explain the results of this study. For a full understanding of the adaptive changes in $GABA_A$ receptor mediated inhibition during epileptogenesis it will be necessary to characterise the changes in receptor density and subunit expression in local brain regions and neuronal populations and to establish their contribution to the increase in β-activity of the EEG, measured in the cortex.

8.5 Conclusion

In summary, in this study it is shown that status epilepticus and/or the ensuing epileptogenic process causes a selective modification in a specific population of $GABA_A$ receptors, which suggests an alteration in subunit composition of the receptor. Furthermore, the stimulus-response relationship of GABAergic modulators is shown

to be altered by epilepsy, which points to an alteration of the neuronal circuits, which generate the observed EEG-effects upon binding of the ligands, as result of epileptogenesis.

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