Pharmacoresistance in epilepsy : modelling and prediction of disease progression

Liefaard, C.

Chapter VI

Changes in $GABA_A$ receptor properties in amygdala kindled animals: *in vivo* studies using [^{11}C]flumazenil and positron emission tomography

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Summary

Purpose The purpose of the present investigation was to quantify alterations in $GABA_A$ receptor density *in vivo* in rats subjected to amygdala kindling.

Methods The $GABA_A$ receptor density was quantified by conducting a [^{11}C]flumazenil PET study according to the full saturation method,[1] in which each animal received a single injection of flumazenil to fully saturate the $GABA_A$ receptors. Subsequently, the concentration-time curves of flumazenil in blood (using HPLC-UV or LC/MS/MS) and brain (with PET-scanning) were analysed by population modelling using a pharmacokinetic model, containing expressions to describe the time course of flumazenil in blood and brain.

Results The $GABA_A$ receptor density (B_{max}) in kindled rats was decreased by 36% compared with controls. This is consistent with a reduction of 28% in EEG effect of midazolam in the same animal model,[2] suggesting that a reduced number of $GABA_A$ receptors underlies the decreased efficacy of midazolam. Furthermore, receptor affinity (K_D) was not changed, but the total volume of distribution in the brain (V_{Br}), is increased to 178% of control after kindling, which might indicate an alteration in the transport of flumazenil across the blood-brain barrier.

Conclusions Both the $GABA_A$ receptor density (B_{max}), and possibly also the blood-brain barrier transport of flumazenil (V_{Br}) are altered after kindling. Furthermore, this study shows the feasibility of conducting PET studies for quantifying moderate changes in $GABA_A$ receptor density in a rat model of epilepsy *in vivo*.

6.1 Introduction

In chronic epilepsy, about 20-40% of all patients develop resistance to anticonvulsant drug treatment during the course of their disease, with the underlying mechanisms

remaining unclear.[3,4] Because pharmacoresistance occurs in nearly all types of seizures and epileptic syndromes, several mechanisms may be involved in the development of pharmacoresistance. Currently, two hypotheses are considered most important: 1) the drug target hypothesis, which postulates that alterations of drug targets in the brain lead to reduction or elimination of drug responsiveness, and 2) the multidrug transporter hypothesis, assuming that limited access of antiepileptic drugs to the brain is a result of overexpression of multidrug efflux transporters at the blood-brain barrier.[5,6]

The $GABA_A$ receptor is target for various antiepileptic drugs.[6] In temporal lobe epilepsy, a type of epilepsy known for a high prevalence of pharmacoresistance, both decreased binding and reduced effect of GABAergic drugs have been demonstrated. For instance, both in animals and humans, binding of radioactive labelled flumazenil has been shown to be decreased.[7–9] Studies with midazolam in different animal models, showed that the facilitatory action of this drug on $GABA_A$ mediated inhibition is moderately reduced after amygdala kindling, cortical stimulation and in animals with genetic absence epilepsy,[2] and more strongly after induction of status epilepticus by kainic acid.[10] Furthermore, Jones and colleagues showed that diazepam or phenobarbital were very effective in terminating pilocarpine-induced status epilepticus in rats when injected only a few minutes after the first motor seizure. However, these drugs were less effective at later time points, suggesting a progressive decrease in GABAergic effect as result of continuing status epilepticus.[11]

An important question in this respect is, whether the observed decrease in GABAergic effects is caused by a reduction in the number of $GABA_A$ receptors over time. To address this issue, it would be advantageous to study the properties of this receptor *in vivo*. Due to its non-invasive nature, positron emission tomography (PET) is an attractive technique to study receptor characteristics *in vivo*.[12] Moreover, Bouvard *et al* showed that PET is an interesting tool for investigating epilepsy induced short term modulations of the $GABA_A$ receptor.[13] Using [^{11}C]flumazenil and PET, they twice measured B'_{max} in 10 epileptic patients and 10 normal controls, at a one week interval. 50% of the patients, but no controls, demonstrated clinically significant interscan variations in B'_{max} in the mesial temporal region. Moreover, the value of B'_{max} was shown to be correlated to the duration of the interictal period. To date, high resolution PET scanners are available, which can be used for studies in small animals. This facilitates integration of mechanistic studies in experimental animal models with clinical studies in humans.

Recently, we reported a novel full-saturation method, in which the whole range of receptor occupancies, obtained in a single experiment, was used to calculate both $GABA_A$ receptor density (B_{max}) and affinity (K_D).[1] After injection of an excess amount of [^{11}C]labelled flumazenil to fully saturate the receptors, the concentration-time curves of flumazenil in blood and brain were measured. The data were analysed by population pharmacokinetic (PK) modelling, providing simultaneous estimates of both B_{max} and K_D. Population (or mixed-effects) modelling implies that data of a whole population

are analysed simultaneously, whilst taking into account interindividual variability in parameter values by using stochastic models.[14]

In the present study, possible alterations in the properties of the $GABA_A$ receptor in experimental epilepsy were studied in vivo. As shown by Cleton *et al*, the maximal EEG-effect of midazolam in kindled rats is reduced to 72% as compared to controls.[2] It has been hypothesised that this reduction in midazolam efficacy is caused by a decrease in receptor density. To investigate this hypothesis *in vivo*, in the present study PET scans with [^{11}C]flumazenil were performed according to the full saturation method, estimating B_{max} and K_D in rats subjected to amygdala kindling and in control rats. The kindling model was chosen, because there is far less inter-animal variation than with other models, such as the post-status epilepticus models. Moreover, if an alteration in $GABA_A$ receptor properties can be quantified in this mild animal model, it would demonstrate the feasibility of the method to detect receptor changes at an early stage in progressive animal models. This might also facilitate the design of translational studies in man.

6.2 Methods

6.2.1 Animals

Adult male Wistar 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 Leiden University.

6.2.2 Experimental setup

In total 22 rats were used, from which 12 animals were subjected to amygdala kindling, and 10 rats received sham stimulations.

For kindling a bipolar electrode was implanted in the right basolateral amygdala (2.5 mm posterior and 5.4 mm lateral from bregma, and 7.8 ventral from the brain surface). This surgery was performed 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. The electrodes consisted of twisted, 150 μm insulated stainless steel wires (E363/3, Plastics One, Roanoke, Virginia, USA). The tips were vertically separated by 500 μm. A skull screw (11 mm anterior, 2.5 mm lateral from lambda) served as the reference electrode. 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 before kindling was started. Rats of the kindling group were kindled twice daily until fully kindled. Control rats were handled identically, but not stimulated.

One week after the last stimulation session, 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

Table 6.1: Means of exact individual dosages, and number of animals per dose group.

Dose group	First study[a b] (controls)	Second study[a] (controls)	Second study[a] (kindled)
1	0.8 ± 0.1 μg (n = 6)	0.2 μg (n = 1)	1.4 ± 1 μg (n = 2)
25	26 ± 2 μg (n = 2)	23 ± 0.3 μg (n = 2)	22 ± 0.5 μg (n = 2)
50	47 ± 2 μg (n = 3)	39 ± 0.7 μg (n = 2)	41 ± 6 μg (n = 3)
100	89 ± 2 μg (n = 3)	91 ± 3 μg (n = 2)	91 ± 5 μg (n = 3)
500	477 ± 15 μg (n =7)	439 ± 28 μg (n = 3)	488 ± 8 μg (n = 2)
1000	919 μg (n = 1)	NA[c]	NA[c]
2000	1,753 ± 3 μg (n = 2)	NA[c]	NA[c]

[a] Mean of exact individual doses ± SD. Between brackets number of animals per dose group.
[b] Three animals were scanned twice at an interval of 2 days after administration of the following dose combinations (in order of administration): 474 and 1.06 μg, 462 and 919 μg 1,750 and 0.63 μg. Thus in total 21 animals were used, resulting in 24 studies. See for further details.[1]
[c] Not available.

base (Ketalar, Parke-Davis, Hoofddorp, The Netherlands, 1 μg/g body weight, subcutaneously) and medetomidine hydrochloride (Domitor, Pfizer, Capelle a/d IJssel, 0.1 μg/g body weight, intramuscularly).

After two days of recovery, [^{11}C]flumazenil studies were performed using the recently reported full saturation method.[1] Different dose groups were used, which will be indicated using the approximate values of 2000, 1000, 500, 100, 50, 25, and 1 μg. In table 6.1 the measured dosages of flumazenil and the number of animals in each dose group are given. To increase accuracy and precision of parameter estimation, recently published control data[1] were also included. The characteristics of all data are summarised in table 6.1.

6.2.3 Kindling procedure

First, the threshold for afterdischarges was determined for each individual rat in the following manner: rats were stimulated at 5 minutes intervals, starting at 25 μA (2 s, 50 Hz, 2 ms bipolar pulse train), increasing the intensity by 50 μA increments until an afterdischarge of at least 1 s occurred. Thereafter the animals were kindled twice daily at 150 μA above their individually determined threshold until fully kindled, meaning that on 6 sequential kindling session class V seizures had occurred. Seizures were classified according to Racine's scale.[15]

6.2.4 *Production of [^{11}C]flumazenil*

[^{11}C]Flumazenil was produced according to the method as described by Maziere *et al*,[16] yielding a solution of 8–12 GBq of [^{11}C]flumazenil in a mixture of 1 ml of ethanol and 14 ml of 7.1 mM NaH_2PO_4 solution in saline with a specific activity of 33–850 GBq/μmol. Both chemical and radiochemical purity proved to be >99.8%.

6.2.5 *PET-scanning*

Animals were scanned according to the protocol as reported previously.[1] All PET data were acquired using a double LSO-layer high-resolution research tomograph (HRRT; CTI, Knoxville, TN), which was designed for small animal and human brain studies. The resolution of this scanner is approximately 2.5 mm in all directions, while the absolute point-source sensitivity is as high as 7%. The performance characteristics of the scanner have been described elsewhere.[17,18]

First, a transmission scan was acquired using a 740 MBq ^{137}Cs point source. Subsequently, PET data acquisition was started simultaneously with the injection of 37 MBq [^{11}C]flumazenil mixed with unlabelled flumazenil in the amounts as given in table 6.1. PET data were acquired during 30 minutes, the duration being based on the metabolic half-life of FMZ ($t_{1/2}$ = 8.3 min[19]). All data were acquired in 64 bits list mode. After acquisition, list mode data were converted to 16 sinograms with frame durations increasing from 15 up to 300 seconds and reconstructed using an OSEM 3D algorithm, a matrix size of 256 × 256 × 207 and a cubic voxel size of 1.21 × 1.21 × 1.21 mm^3. This algorithm was chosen, because it ensures optimal usage of the scanner resolution and avoids difficulties with data inhomogeneities.[20] During reconstruction data were normalised and corrected for attenuation, dead time, randoms and scatter using the transmission scan.

During the experiment 11 blood samples (100 μl) were taken at t = 1, 2, 3, 5, 7, 10, 12, 15, 20, 25, and 30 minutes after injection, and immediately diluted with 0.5 ml 0.42% NaF in water at 0 °C to inhibit esterase activity and thus prevent flumazenil metabolism in the blood sample. Immediately after the experiment, samples were stored at -80 °C until the time of analysis.

6.2.6 *Drug analysis in blood*

Flumazenil concentrations in blood were analysed using a high-performance liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) method. Firstly, 10 μl 500 ng/ml 1'-hydroxymidazolam (Toronto Research Chemicals Inc., North York, Canada) was added as internal standard. Subsequently flumazenil and the internal standard were extracted with liquid-liquid extraction. The samples were diluted with 500 μl of tetraborate pH standard solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). This mixture was extracted with 5 ml ethyl acetate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After centrifugation, 4 ml of the organic phase was transferred into a clean tube, and evaporated to dryness under a gentle stream of nitrogen gas. The residue was reconstituted with 250 μl mobile phase, from which 10 μl was injected into an 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 40 °C (Shimadzu, Kyoto, Japan), an Inertsil Ph-3 column (5 μm, 150 mm, 2.1 mm; GL Sciences Inc., Tokyo Japan), and an API3000 mass-spectrometer (AB/MDS Sciex Instrument, Foster City, 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 23 and 34 V for flumazenil and 1'-hydroxymidazolam respectively. The mobile phase consisted of a mixture of

10 mM ammonium acetate (pH=4.0; MP-A), and methanol containing 0.2% acetic acid (MP-B) under gradient conditions: at start of the analysis, the ratio of MP-A:MP-B was 70:30, changing to 20:80 at 2 minutes after start. Flow rate was 0.2 ml/min, and run time 6 minutes. Flumazenil (m/z 304.1→258.1) and 1'-hydroxymidazolam (internal standard, m/z 342.2→203.1) were detected using a selected reaction monitoring mode. Concentrations of flumazenil were determined from the peak area ratios of flumazenil *versus* internal standard. The limit of quantification was 0.5 ng/ml. Linear calibration curves were obtained in the range of 0.5–1,000 ng/ml. The intra-day assay coefficient of variation at 1.5, 50.0, and 800.0 ng/ml were 2.8, 3.7, and 3.6% ($n = 5$) respectively, and accuracy were 5.1, 3.5, and 4.9% ($n = 5$) respectively. Data acquisition and data analysis was performed using the software package Analyst (version 1.3, AB/MDS Sciex Instrument, Foster City, CA, USA).

6.2.7 Data analysis

In order to determine the activity-time curve of flumazenil in brain tissue, a region of interest (ROI) was placed over the cortex using the Vinci software package (version 1.75.0, Max-Planck Institute for Neurological Research Cologne, Köln, Germany). The position of this ROI was defined visually on frame 5 (i.e., 60–75 s post injection, at the peak of the concentration-time curve.[1] This ROI was projected onto all dynamic frames, which, after decay correction, resulted in an activity-time curve of [^{11}C]flumazenil. Using the specific activity of the injected [^{11}C]flumazenil, the concentration-time curve of total flumazenil in brain was obtained, as described previously.[1]

Data were 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.[14] In brief, population analysis results in estimates of three types of parameters: 1) structural model parameters, which are average values for the population of parameters describing the dose-concentration-effect relationships, 2) inter-individual (IIV) variances and covariances of the structural model parameters, and 3) residual variance, which is associated with measurement/intra-individual error.[14]

The concentration-time profiles of flumazenil in blood and brain were analysed with the four-compartment model described previously,[1] comprising a blood, a tissue and two brain compartments. A schematic representation of the model is shown in figure 6.1. Upon administration into the blood compartment, flumazenil can distribute to the tissue and the brain free compartment, and in the brain it then can specifically bind to the $GABA_A$ receptor, which is represented by the brain bound compartment. The concentration-time profile, obtained by PET is described by the total concentration in both brain compartments ("Brain Free" and "Brain Bound"). Using this method, the volumes of distribution of the blood, tissue and two brain compartments (V_C, V_T, and V_{Br} respectively) are estimated, as well as the intercompartmental clearances to describe distribution to tissue and brain (Q, and Q_{Br} respectively) and the total clearance out of the body (CL). The clearances are calculated from the rate constants between the compartments and the volumes of distribution ($CL = k_{10} \cdot V_C$, $Q = k_{12} \cdot V_C = k_{21} \cdot V_T$, $Q_{Br} = k_{13} \cdot V_C = k_{31} \cdot V_{Br}$[1]). The specific binding to the $GABA_A$ receptor is described in terms of B_{max}, k_{on}, and k_{off}, which are all estimated. The dissociation rate constant K_D is calculated from k_{on} and k_{off}.

The residual error was assumed to be proportional to the concentration in blood and brain (σ^2_{blood} and σ^2_{PET} respectively). In the previous study, an additive residual error was used to take

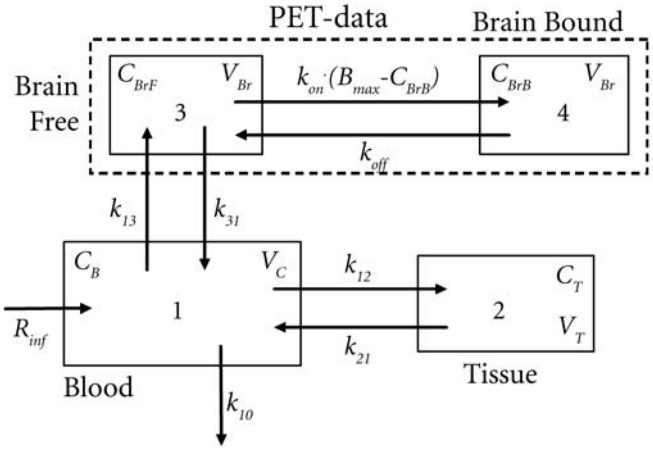

Figure 6.1: The structural 4-compartment PK model. C_B, C_T, C_{BrF}, C_{BrB} are concentrations of flumazenil in blood, tissue, brain free and brain bound compartments, respectively. R_{inf} is the zero-order administration rate. V_C, V_T, V_{Br} are pharmacokinetic volumes of distribution of blood, tissue and brain compartments, respectively; k_{12}, k_{21}, k_{13}, k_{31} describe exchange between compartments; k_{on}, k_{off}, B_{max} describe the association rate, the dissociation rate and specific binding; k_{10} describes total elimination from the body (adapted from Liefaard *et al*,[1] with kind permission of Springer Science and Business Media).

into consideration the greater uncertainty in blood concentrations that are close to the detection limit. The value of this additive residual variance was fixed at 25, which is the square of half of the detection limit (detection limit: 10 ng/ml,[1]). In the present study, the detection limit for flumazenil concentrations in blood was only 0.5 ng/ml, thus only a few data points were near the detection limit. Therefore, it was not necessary to include an extra residual error in the model, as it did not improve the fit.

The effect of kindling on any parameter describing the concentration-time profiles of flumazenil in blood and brain was studied by estimating a fractional difference between the parameter values for the control versus the kindled animals:

$$P_{KI} = (1 + \alpha) \cdot P_{control} \tag{6.1}$$

in which P_{KI} and $P_{control}$ represent the estimate of the inspected parameter for the kindled, or control animals respectively, and α the fractional difference between these parameter estimates. This relation was chosen for statistical reasons, because if a value of α is significantly different from 0, this implies that P_{KI} and $P_{control}$ are different.

6.2.8 Simulations

To study whether the kindling-induced effects on B_{max} and V_{Br} were causally related, simulations were performed using the software package Berkeley Madonna 8.0 (Macey and Oster, University of California at Berkeley, USA). To this end, the total concentration and the fraction of specifically bound flumazenil in the brain were simulated for control and kindled rats, using the four-compartment model and the parameter values obtained from the analysis of the full dataset. The concentrations simulated were in the full dose range.

Table 6.2: Parameter estimates.

Parameter	Control rats previous study without covariate[ab]	All control rats without covariate[ac]	Control & KI rats covariates B_{max} & V_{Br}[ad]
Structural parameters			
V_C (ml)	38.9 ± 9.1 (21.2–56.6)	27.7 ± 7.5 (13.0–42.4)	23.7 ± 3.0 (17.9–29.5)
V_T (ml)	179 ± 27.4 (125–233)	189 ± 30.1 (130–248)	200 ± 26.5 (148–252)
V_{Br} (ml)	25.4 ± 4.3 (17.0–33.8)	36.2 ± 5.5 (25.3–47.1)	37.8 ± 2.9 (32.2–43.4)
CL (ml/min)	18.9 ± 0.7 (17.4–20.4)	19.6 ± 1.2 (17.2–22.0)	20.9 ± 1.1 (18.8–32.0)
Q (ml/min)	20.3 ± 3.2 (14.0–26.6)	18.4 ± 4.0 (10.6–26.2)	19.4 ± 2.7 (14.1–24.7)
Q_{Br} (ml/min)	26.3 ± 5.7 (15.1–37.5)	26.1 ± 7.9 (10.7–41.5)	27.5 ± 4.8 (18.1–36.9)
B_{max} (ng/ml)	14.1 ± 5.5 (3.3–24.9)	20.1 ± 9.0 (2.4–37.8)	25.4 ± 3.6 (18.4–32.4)
K_D (ng/ml)	4.6 ± 2.3 (0.022–9.1)	6.7 ± 4.0 (-1.2–14.5)	7.6 ± 1.1 (5.5–9.6)
$\alpha(B_{max})$	NA[f]	NA[f]	-0.36 ± 0.092 (-0.54–−0.18)
αV_{Br}	NA[f]	NA[f]	0.78 ± 0.12 (0.55–1.0)

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6.2.9 *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 inter-individual variability (IIV) for any parameter was based on three criteria. 1) The value of objective function, which is a measure of the goodness of fit (approximately -2 times log likelihood) had to be significantly lower for the model with IIV included, compared to the model without inclusion of IIV. In this study a significance level of $p < 0.001$ is used, which is related to a difference between the value of objective function between two models (Δobj.f.) of at least 10.8, based on a Chi-squared distribution of Δobj.f. 2) The variability had to be significantly different from zero. 3) Inclusion should not affect estimation of any other parameter.

A parameter was considered significantly different between control and kindled animals, if

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Parameter	Control rats previous study without covariate[ab]	All control rats without covariate[ac]	Control & KI rats covariates B_{max} & V_{Br}[ad]
Inter-individual variability[e]			
ω^2 (Q_{Br})	NA[f]	0.23 ± 0.081 (0.074–0.39)	0.23 ± 0.057 (0.12–0.34)
Residual errors[h]			
σ^2_{blood}	0.16 ± 0.027 (0.10–0.21)	0.21 ± 0.032 (0.14–0.27)	0.26 ± 0.035 (0.19–0.33)
σ^2_{PET}	0.23 ± 0.033 (0.17–0.30)	0.23 ± 0.033 (0.17–0.30)	0.21 ± 0.031 (0.15–0.27)

[a] Parameter estimate ± SE. Between brackets 95% confidence interval. These values indicate the precision of estimation of the parameters, rather than variability within the population.
[b] Data from previous study.[1]
[c] Data from previous study[1] and control rats of present study simultaneously analysed.
[d] All data simultaneously analysed. If a parameter is significantly different between control and kindled animals, the fractional difference is given for that parameter ($\alpha(B_{max})$ and $\alpha(V_{Br})$).
[e] Inter-individual variability (IIV, ω^2) was found to be significantly greater than zero for Q_{Br} (intercompartmental clearance between blood and brain compartment) only. This indicates that this parameter can be different between individuals of the population.
[f] Not available.
[h] The residual error (σ^2), which account for any measurement/intraindividual error, is assumed to be proportional to the concentration in either blood, or brain.

including the factor α resulted in Δobj.f. ≥ 10.8 ($p < 0.001$), and α was significantly different from zero.

6.3 Results

To obtain the most accurate and precise estimates of all parameters, previously published control data[1] were also included in the present study. As shown in table 6.2 the parameter estimates for control rats of both studies are similar, indicating that possible differences between both studies had no effect on the PK of flumazenil. In this table the parameter estimates are given, with their standard errors (SE) and corresponding confidence intervals.

All kindled animals reached class V seizures within 5–20 sessions, as characterised by behavioural clonic convulsions with rearing and falling.[15] Stimulation was continued until generalised seizures had been elicited in 6 sessions, which required a mean of

Table 6.3: Values of objective function.

Model	Without covariate[a]	Covariate B_{max}[a]	Covariate V_{Br}[a]	Covariate B_{max} & V_{Br}[a]
Value of objective function	10673	10661	10625	10619
Δobj.f.[b]	NA[c]	12	48	54

[a]Complete dataset with both control and kindled rats was used.
[b]Since the difference between the value of objective function (Δobj.f.) is Chi-squared distributed, at a significance level of $p < 0.001$, a difference of at least 10.8 is significant.
[c]Not available.

19 sessions (range 10–25). This observed rate of kindling is consistent with previously reported data.[2, 21]

Simultaneous analysis of all animals resulted in the parameter estimates, presented in table 6.2. Next, it was tested for each parameter whether it was significantly different between control and kindled animals. For those parameters where this was the case the parameter estimate for the control animals and the fractional difference between the control and kindled animals are reported. Thus, after kindling, receptor density was reduced with 36% ($\alpha(B_{max})$ = -0.36 ± 0.092), and the total volume of distribution in the brain was increased with 78% ($\alpha(V_{Br})$ = 0.78 ± 0.12) compared to control. In table 6.3 the values of objective function for the different models with or without the effect of kindling on B_{max} and/or V_{Br} are shown. This indicates that implementing a fractional difference for either B_{max} or V_{Br} between kindled and control animals results

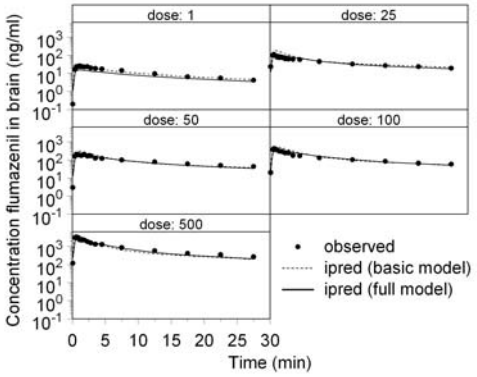

Figure 6.2: Concentration time profiles of flumazenil in the brain of kindled rats. The dots represent measured concentrations, the solid lines represent the individual predictions by the final model, in which the effects of kindling on the parameters B_{max} and V_{Br} are included, whereas the broken lines represent the individual predictions of the model without these effects. Typical examples were used for each dose group.

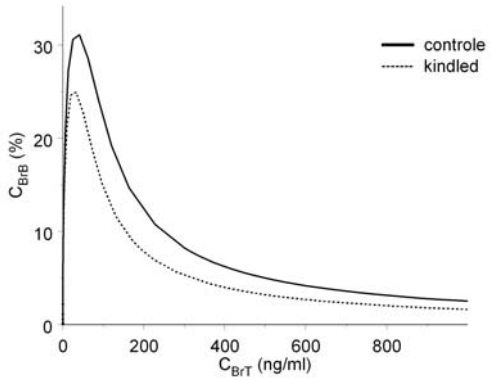

Figure 6.3: Simulations to assess whether there was a correlation between a kindling induced decrease in B_{max} and increase in V_{Br}. C_{BrB} is the concentration flumazenil specifically bound to the $GABA_A$ receptor, presented as percentage of the total flumazenil concentration in the brain, which is represented by C_{BrT}. The solid line represents the simulations for control rats, and the broken line represents the simulations for kindled rats.

in a significant drop in the value of objective function. This value is even further reduced when fractional differences for both parameters were implemented simultaneously. Thus, kindling significantly affected both B_{max} and V_{Br}.

In figure 6.2 typical examples of measured concentration-time profiles of flumazenil in the brain of the kindled rats are shown for each dosing group, with concentration-time curves as predicted by both models without and with kindling effect on B_{max} and V_{Br}. This figure shows that the latter model indeed describes the data more precisely, mainly at the lower concentrations. This was to be expected, because at the lower concentrations, the fraction of flumazenil specifically bound to the $GABA_A$ receptor is higher. This is illustrated by figure 6.3, in which the fraction of specifically found flumazenil in the brain ($\%C_{BrB}$) was simulated as function of the total brain concentration (C_{BrT}) for control and kindled rats. Thus, the greatest impact of an alteration in B_{max} and V_{Br} may be expected at the lower concentration levels of flumazenil.

6.4 Discussion

In the present study, the maximal binding capacity of flumazenil to the $GABA_A$ receptor, as represented by the parameter B_{max}, is shown to be decreased in rats subjected to amygdala kindling. This is consistent with reported reductions in flumazenil binding in epileptic patients and animal models of temporal lobe epilepsy.[7–9] The currently obtained decrease in B_{max} by 36% in kindled animals as compared to controls corresponds well with a reduction of 28% in maximal EEG effect of midazolam, which was observed in the same animal model.[2] This indicates that a reduction in maximal binding capacity of the $GABA_A$ receptor may underlie the reduced EEG-effect of midazolam in kindled rats.

In contrast, the affinity for the receptor, as expressed by K_D, was not affected, which is consistent with the data of Cleton *et al*, who found that the EC_{50} was not altered after kindling.[2]

The ability to detect a decrease in receptor binding in the amygdala kindling model, which is a mild animal model for epilepsy, demonstrates that conducting a PET-study according to the full saturation approach is a sensitive method for detection of moderate reductions in $GABA_A$ receptor density. Recently, a severe and progressive decrease in midazolam EEG effectivity in the post-status epilepticus rat model of epilepsy was reported.[10] *Ex vivo* studies suggested a correlation between the level of reduction in midazolam EEG effectivity and the amount of $GABA_A$ receptor binding in the brain.[10] At the same time, as the decrease in EEG effectivity was more pronounced than the decrease in $GABA_A$ receptor binding, the data suggested that multiple mechanisms are responsible for the decrease in EEG effectivity. Using this PET-approach role and extent of the reduction in $GABA_A$ receptor can be studied *in vivo* longitudinally.

Quite unexpectedly, electrical kindling of the amygdala affected the total distribution volume of flumazenil in the brain (V_{Br}) as well, by increasing the value of V_{Br} to 178% of control. The possible mechanisms underlying the increase of V_{Br} deserve further discussion. As shown previously, V_{Br} is the hypothetical volume in the brain, containing all flumazenil available in the brain, which might be either specifically bound to the $GABA_A$ receptor, or non-specifically bound, or free.[1] An increase in V_{Br} therefore suggests lower concentrations of flumazenil in the brain. Theoretically, several explanations are possible for this finding. Firstly, there can be reduced uptake of flumazenil in the brain by either a decrease in transport into the brain, or an increase in transport out of the brain. Secondly, non-specific binding might be decreased. Finally, it can reflect reduced specific binding of flumazenil to the $GABA_A$ receptor as reflected by an alteration in B_{max} and/or K_D.

The binding of flumazenil to the $GABA_A$ receptor is reduced after kindling, as this study shows that the maximal binding capacity (B_{max}) is decreased to 36% of control values. This might imply that the increase in V_{Br} simply reflects the decrease in B_{max}, and that these parameters are correlated. In that case, the fraction of specifically bound flumazenil in the brain as function of the total brain concentration would coincide for control and kindled rats. However, simulations showed that the fraction of flumazenil which is specifically bound to the $GABA_A$ receptor is smaller for kindled rats (figure 6.3), implicating that the kindling-induced decrease in B_{max} is not the underlying mechanism responsible for the increase in V_{Br}.

As discussed previously, with the current setup it is not possible to discriminate between non-specifically bound and free flumazenil in the brain.[1] Thus, strictly speaking, it is not possible to determine whether transport of flumazenil to the brain, or the non-specific binding in the brain is altered. However, flumazenil is known to have low non-specific binding,[22] suggesting that a decrease in non-specific binding might not be the most important mechanism responsible for the observed increase in V_{Br}.

Alternatively, the transport of flumazenil across the blood-brain barrier might be altered as result of kindling. Different groups have reported an upregulation of multidrug transporters in epilepsy, which may result in an increased transport out of the brain, and therefore lower brain concentrations.[23,24] Moreover, studies in various animal models of epilepsy, demonstrate that the antiepileptic drugs oxcarbazepine or phenytoin are more effective in suppression of seizure activity when an inhibitor of Pgp or MRP is coadministrated.[25,26] On the other hand, Seegers *et al* found that amygdala kindling does not induce any lasting overexpression of Pgp in several brain regions.[27] Furthermore, Mahar Doan *et al* showed that flumazenil is not a substrate for Pgp,[28] thus arguing against this explanation.

However, other multidrug transporters may be involved. For instance, recently a non-ABC multi-specific transporter, RLIP76, has been shown to be upregulated in brain samples from epileptic patients. The importance of this transporter in transport of carbamazepine was studied *in vitro*. Using anti-RLIP76 antibodies the efflux of carbamazepine was decreased by 74%, whereas anti-MDR1 antibodies inhibited this transport only by 13%.[29] These results suggest that a role for other multidrug transporters in the transport of flumazenil across the blood-brain barrier is a reasonable possibility. Whether this indeed the case remains to be demonstrated.

6.5 Conclusion

In summary, the $GABA_A$ receptor density is reduced in amygdala kindled rats, as represented by a decrease in B_{max}. This may underlie the reduced efficacy of midazolam in these animals. Furthermore, it seems that blood-brain barrier transport of flumazenil is also altered, as the total volume of distribution in the brain (V_{Br}) is increased. Thus, conducting a flumazenil PET study according to the full saturation approach is a sensitive method to quantify changes in $GABA_A$ receptor properties and pharmacokinetics of flumazenil *in vivo* in an animal model of epilepsy. This clearly shows the usefulness of these studies in epileptogenesis and pharmacoresistance development using chronic epilepsy models, which may be extended to clinical studies.

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