Pharmacoresistance in epilepsy : modelling and prediction of disease progression

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Chapter IX

Modelling and prediction of epilepsy progression and pharmacoresistance development: Summary, conclusions and perspectives

9.1 Introduction

The investigations in the present thesis focussed on the relationship between time course of epileptogenesis and associated changes in GABAergic inhibition, leading to pharmacoresistance. Despite the development of several new antiepileptic drugs, pharmacoresistance is still an unresolved problem in epilepsy. For instance, in about 60% of people suffering from mesial temporal lobe epilepsy (mTLE) seizures are only poorly controlled by pharmacotherapy.[1,2] As mTLE has a relatively high prevalence compared to other pharmacoresistant epilepsies and as several animal models are available for this form of epilepsy, most studies on pharmacoresistance focus on mTLE.

Two main mechanisms underlying development of pharmacoresistance have been proposed: (a) the multidrug-transporter hypothesis, suggesting that over expression of multidrug-transporters at the blood-brain barrier limits the access of antiepileptic drugs to the brain and (b) the drug target hypothesis, which says that alterations of drug targets in the brain lead to reduced or absent sensitivity to antiepileptic drugs.[3,4] Several studies have shown that multidrug-transporters are indeed up regulated in epilepsy, indicating the validity of the first concept.[5,6] However, alterations in drug targets often have been reported as well.[7,8,4] One of the drug targets, which has been studied extensively, is the $GABA_A$ receptor. The GABAergic system is important for controlling excessive excitatory activation,[9,8] and therefore it is an attractive target for antiepileptic drugs.

In the present thesis, it was hypothesised that alterations in drug targets, leading to pharmacoresistance, originate shortly after start of epileptogenesis, preceding or starting with the first epileptic insult. To test this hypothesis, studies were performed in animal models of epilepsy, using positron emission tomography (PET), electroencephalography (EEG), cortical stimulation and behavioural analysis as endpoints. Moreover, population pharmacokinetic-pharmacodynamic (PK-PD) modelling techniques were used for analysis of the relationships between exposure, or plasma concentrations, and response.

9.2 Biomarkers of disease progression in epilepsy

9.2.1 Selection of biomarkers

In animal models of progressive epilepsy, epileptogenesis can be initialised by a status epilepticus, which is followed by a silent period. To test the hypothesis that

pharmacoresistance originates shortly after the start of epileptogenesis, it is important that biomarkers quantify disease progression already during the silent period. Therefore, two biomarkers were selected, meeting this criterion: 1) the threshold for epileptic seizures, measured by electrical cortical stimulation and 2) quantitative analysis of continuously recorded EEG using quantification of interhemispheric synchronisation based on detection of local maxima or events.

When stimulating the cortex according to the protocol as described by Voskuyl *et al*[10] a convulsion is evoked. This convulsion is characterised by a typical pattern of behavioural components that occur during stimulation and can be controlled by the increment in strength per stimulus and the duration of the stimulation. Based on the latter property, thresholds for eliciting certain components have been defined, such as the threshold for clonic activity (TLS). It has been shown that administration of antiepileptic drugs increased the TLS in a concentration dependent manner,[11–13] suggesting that measurement of the TLS is a good predictor for quantifying excitability of the brain. As the brain is presumed to become more susceptible to seizure induction during epilepsy development, the TLS was expected to decrease after status epilepticus induction, at least at the end of the silent period. The results described in Chapter 3, however, show that the threshold did not decrease, but rather increased, even at four weeks after status epilepticus, at which time the animals showed spontaneous seizures. Therefore, it was concluded that the TLS is not a convenient biomarker for monitoring epilepsy progression and predicting which animals will indeed develop epilepsy.

More promising is quantitative analysis of cortical EEG, based on a method reported by Quian Quiroga *et al*.[14] For this purpose, events were defined in the EEG as local maxima, having an amplitude above a threshold of 4 times the standard deviation of the baseline recording. These local maxima or events often, but not always, are epileptiform discharges. Events were scored on cortical EEG recordings derived from both hemispheres, and the rate at which events occurred, event interval and interhemispheric synchronisation of events were quantified (Chapter 4). Analysis of EEG recorded during status epilepticus showed that many events occurred, resulting in a large increase in event rate. Moreover, events were highly synchronised interhemispherically. Application of the method to a four week recording in a rat which had experienced a status epilepticus showed that even at lower values of event rate, these events were highly synchronised. In control animals, however, only a few events were detected, which were only poorly synchronised. These results strongly suggest that this quantitative method of analysing EEG is a sensitive and selective method, which might yield very promising biomarkers for monitoring progression of epilepsy.

9.2.2 Convulsive threshold in epileptic animals

Although the convulsive threshold appeared not to be a convenient biomarker for monitoring progression of epilepsy, application of cortical stimulation and measurement of the TLS in animals which had experienced status epilepticus raised some important

questions. Firstly, why did the TLS increase after status epilepticus: is it a paradoxical result of epileptogenesis, or can it be explained by an altered seizure pattern? In the latter case, the validity of the TLS as indicator of seizure susceptibility after status epilepticus induction could be challenged. To address this issue, an evoked seizure at day 28 after status epilepticus was analysed by quantitative behavioural analysis. This showed that clonic activity was still present, and that the sequence of seizure components was unaltered (Chapter 3). As clonic activity is the criterion for the TLS, these results confirm the validity of the TLS as a measure of seizure susceptibility after status epilepticus.

So the question remains: why is the TLS increased after induction of status epilepticus? Several reports have shown that status epilepticus is difficult to initiate when animals are kindled prior to status epilepticus, and that these animals are protected from status epilepticus induced neuronal damage.[15,16] In addition, when prior to status epilepticus the TLS was stabilised by stimulating animals twice daily (which is a phenomenon similar to kindling), only a mild reduction in efficacy of midazolam was found at day 4, but not at day 14 post-status epilepticus (unpublished results). In animals which did not experience cortical stimulation before status epilepticus, however, efficacy of midazolam was time-dependently decreased to 26% of the original value at day 14 after status epilepticus.[17] In figure 9.1, these results are visualised by plotting the individual values for E_{max} before and after induction of status epilepticus. These results suggest a protective mechanism, which is activated by either single intense (status epilepticus) or repeated moderate seizure activity (kindling or stabilisation of the TLS). This mechanism might explain the increase in TLS after induction of status epilepticus.

The quantitative behavioural analysis was performed primarily to check whether

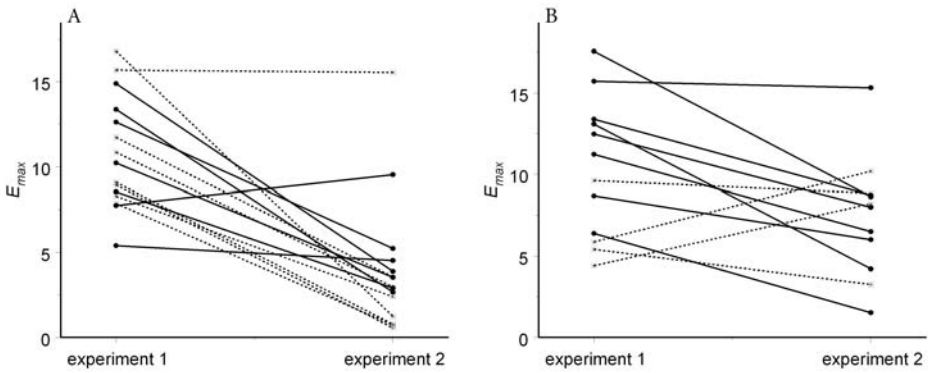

Figure 9.1: Influence of electrical cortical stimulation prior to status epilepticus on epilepsy induced changes in efficacy of midazolam. Depicted are E_{max} values of midazolam in individual animals before (experiment 1) and after (experiment 2) induction of status epilepticus. Experiment 2 was performed either at day 4 (solid lines) or day 14 (dotted lines) after status epilepticus. Panel A shows the results obtained in rats which were not cortically stimulated, whereas rats in panel B were subjected to electrical cortical stimulation prior to the first midazolam experiment.

the TLS was still usable as a pharmacodynamic endpoint, but in addition it showed some quantitative differences in seizure components. Rats which had experienced status epilepticus displayed significantly less clonic activity of forelimbs, eye blinks and rapid movements of the head and/or body, but more tonic hindlimb events as compared to controls (Chapter 3). As reviewed by Velísková,[18] the different seizure components originate from specific brain structures, indicating that a modified seizure pattern points to altered contribution of different brain regions. Although the investigations described in this thesis did not allow to make definite conclusions with regard to which brain structures were affected, results indicate that quantitative behavioural analysis might be a sensitive method to investigate this.

9.2.3 Quantitative analysis of EEG in epileptic animals

Analysis of the EEG recorded during status epilepticus induction showed that four phases could be distinguished, which differed in level of event rate and interhemispheric synchronisation (Chapter 4). In the present investigations, epileptic behavioural manifestations were not continuously recorded in a quantitative manner, but interestingly, qualitative observations showed that the EEG phases were accompanied by specific epileptic behaviour. Therefore, it is expected that EEG events and their synchronisation are closely correlated to behavioural motor seizures, the more so, because cortical EEG was used. In that case, this method for quantification of EEG events and their synchronisation offers an interesting opportunity to automatically detect motor seizures.

Furthermore, analysis of the four week EEG recording in a kainic acid-treated rat using the synchronisation method showed that induction of status epilepticus resulted in increased event rate and synchronisation for about two days, where after both parameters dropped even below control levels. From day 6 to 12 temporary increases in event rate were observed, and finally at day 12 the event rate started to increase slowly, but continuously. Interestingly, events observed in the kainic acid-treated rat were strongly synchronised, as an increase in event rate was parallelled by an increase in synchronisation. In contrast, a four week recording in a control rat showed no remarkable changes in any of the parameters over time. Although a few events were observed in this rat, these events were not synchronised, as shown by a constant low level of synchronisation.

The observed differences in time course of EEG events, in conjunction with their synchronisation, between a kainic acid-treated and a control rat, indicate that this analysis is able to discriminate between EEG events in epileptic *versus* control animals. To obtain a quantitative method for automatically monitoring disease progression, differences in EEG response between controls and epileptic animals should be studied in a larger group of animals, and a quantitative measure should be defined. Based on present results, it is expected that an elevated event rate, parallelled by an increase in synchronisation, might be a useful candidate parameter for this purpose.

9.3 The $GABA_A$ receptor in epilepsy

9.3.1 Quantification of receptor expression in vivo

Positron emission tomography (PET) is an attractive technique to investigate receptor properties *in vivo*. Different methods have been developed for quantification of the $GABA_A$ receptor using the [^{11}C]labelled GABAergic antagonist flumazenil as a ligand.[19–24] A major drawback of these methods is that to obtain estimates of B_{max} and K_D separately, multiple scans have to be performed. Therefore, a new method was developed, by which B_{max}, K_D and blood-brain barrier transport were quantified in a single PET experiment per animal, as presented in Chapter 5. This novel method was based on the use of the complete range of receptor occupancies and application of population PK-PD modelling concepts. As flumazenil is an antagonist of the $GABA_A$ receptor without pharmacologic or toxicologic effects, it was possible to administer a saturating dose. Moreover, flumazenil is very rapidly cleared from the body, as the half-life of flumazenil in the rat is only 8.3 minutes.[25] This means that during a scan of 30 minutes nearly the whole range of receptor occupancies between 100 and 0% is covered.

Quantification of receptor expression using this full saturation method has some important advantages above existing methods. Firstly, as discussed above, only a single experiment is needed to independently determine both B_{max} and K_D. Furthermore, the use of population modelling makes it possible to still include individuals with incomplete data, such as animals receiving a low dose, resulting in blood concentrations below the limit of quantification. Finally, as the concentration of flumazenil in blood was measured using a detection method other than radioactivity, no metabolite detection was needed.

Obviously, it is important to know whether this novel PET method is sensitive for detecting alterations in receptor properties in epileptic animals. Therefore, animals which were fully kindled by stimulation of the amygdala were scanned according to the novel protocol (Chapter 6). This showed that $GABA_A$ receptor expression was reduced, as represented by a decrease in B_{max} by 36% compared to controls. Moreover, the volume of distribution in the brain, V_{Br}, was increased to 178%, reflecting a decrease in brain concentration and thereby pointing to an alteration in blood-brain barrier transport as well. Interestingly, experiments with midazolam in the same animal model showed that the efficacy of this allosteric GABAergic modulator was only 72% of control.[26] This closely resembles the reported reduction in receptor expression of 36%, suggesting that the reduced midazolam efficacy is caused by a decreased receptor expression.

Apart from giving insight in the mechanism underlying reduced efficacy of midazolam, these results also show the feasibility of conducting PET studies for quantifying alterations in $GABA_A$ receptor density in a rat model of epilepsy *in vivo*. Moreover, as it is possible to perform several PET studies in a single animal, information on the time course of receptor property changes can be obtained, which may give insight into the role of $GABA_A$ receptor expression in epileptogenesis and pharmacoresistance development.

9.3.2 *Functionality of the receptor*

It was hypothesised that alterations in drug target, leading to pharmacoresistance, originate shortly after the start of epileptogenesis, preceding or starting with the first epileptic insult. Therefore, changes in functionality of the $GABA_A$ receptor 4 and 14 days after induction of status epilepticus were investigated in Chapter 7 by performing PK-PD experiments with the allosteric modulator midazolam. The intrinsic efficacy of midazolam, as quantified using the total amplitude of the β-frequency band of the EEG, was decreased in a time-dependent manner, as the maximal effect was reduced to 52% of the original value at day 4 and even to 26% at day 14 after status epilepticus. This indeed shows that already during the silent period the drug target is significantly altered, leading to decreased efficacy of midazolam.

Ex vivo binding studies with [^{3}H]labelled flumazenil in the rat brain showed that this decreased efficacy only partly concurred with a decline in flumazenil binding. This raises the intriguing question as to what could explain the observed discrepancy between moderate reduction in flumazenil binding and more pronounced decrease in midazolam efficacy. As midazolam is an allosteric modulator, a first possibility could be that apart from a reduced expression of the $GABA_A$ receptor, the efficacy of midazolam is reduced because of decreased availability or release of GABA. In Chapter 8 the efficacy of tiagabine after status epilepticus was investigated. Tiagabine inhibits reuptake of GABA, thus resulting in prolonged presence of GABA near the $GABA_A$ receptors. As it was shown that the efficacy of tiagabine was unaltered, it could be concluded that GABAergic inhibition itself remained intact after status epilepticus induction.

Another reason could be that the population of $GABA_A$ receptors reflected by flumazenil binding, and that giving rise to increase of β-activity in the cortical EEG following midazolam administration might not overlap completely. This hypothesis is supported by the observation that the sedating effect of midazolam was not decreased, not even in rats showing almost no EEG response to midazolam administration, suggesting that different $GABA_A$ receptor populations are responsible for EEG effect and sedation.

Finally, several epilepsy induced changes in subunit composition of the $GABA_A$ receptor have been reported.[27,3,28–33] This can differentially affect binding of ligands to the receptor, as most ligands are subunit selective. For instance, it is known that benzodiazepines are highly selective for receptors with γ_2 subunits, thus a loss of this subunit would render the receptor insensitive to midazolam.[34] In contrast, flumazenil binding would not be affected in this case, as flumazenil is far less specific for the subunit composition of the $GABA_A$ receptor. This suggests that subunit alterations may play an important role in the differential effect of status epilepticus on flumazenil binding and midazolam efficacy.

9.3.3 *Involvement of subunit alterations*

To obtain further insight into possible subunit alterations of the $GABA_A$ receptor after induction of status epilepticus the efficacy of the synthetic neurosteroid alphaxalone was

measured. Neurosteroids are known to be powerful allosteric modulators of GABAergic activation of the $GABA_A$ receptor, preferably those containing δ subunits.[35] In contrast, these receptors are insensitive to modulation by benzodiazepines, including midazolam.[34] Moreover, after induction of status epilepticus, complex changes in expression of subunits have been shown *ex vivo*.[27,36,30,33]

Interestingly, the modulating effect of alphaxalone on GABAergic inhibition 14 days after status epilepticus induction was enhanced, rather than impaired, as shown in Chapter 8. Together with the decreased effect of midazolam, accompanied by a smaller reduction in flumazenil binding, and the unaltered effect of tiagabine, this strongly suggests a selective alteration of the $GABA_A$ receptor after status epilepticus, resulting in a receptor which is more sensitive to modulation by alphaxalone, but less sensitive to modulation by midazolam.

9.4 Conclusions and future perspectives

9.4.1 Quantification of pharmacoresistance in epilepsy

In the general introduction, the following definition of pharmacoresistance was proposed: "pharmacoresistance is the inability of an antiepileptic drug to achieve more than 50% seizure reduction at concentrations that do not cause unacceptable adverse effects". An important attribute of this definition is that pharmacoresistance is defined for a single time point, i.e., the time at which the drug is tested, allowing characterisation of the time course of pharmacoresistance development. To achieve this, a biomarker which is able to non-invasively quantify the degree of pharmacoresistance, expressed as the amount of remaining seizure activity, is required.

In the present investigations, two methods were tested for this purpose. One of them, analysis of cortical EEG by quantification of EEG events, defined as local maxima, together with their interhemispheric synchronisation, offer interesting candidate biomarkers (Chapter 4). Though the method has not been validated yet, it was shown that it is able to both distinguish between EEG events in epileptic versus control animals and quantify temporal differences in event rate and synchronisation during and after status epilepticus. Moreover, there were strong indications that the temporal differences during status epilepticus were correlated to behavioural motor seizures, indicating the fact that this method could be used for automatic detection of motor seizures.

Taking together, after defining and validating the exact quantitative measures derived from this EEG analysis, these parameters are expected to be interesting biomarkers for non-invasive quantification of development of epilepsy and pharmacoresistance. This offers the opportunity to correlate changes in drug targets, such as the $GABA_A$ receptor, to the degree of pharmacoresistance development. Moreover, interventions with antiepileptic drugs or neuroprotective agents can be planned and their effects quantified.

9.4.2 *Alterations in GABAergic inhibition*

PK-PD experiments with midazolam, tiagabine and alphaxalone showed that the subunit composition of the $GABA_A$ receptor is altered after status epilepticus (Chapters 7 and 8). In these investigations, the EEG was used as a pharmacodynamic endpoint. Provided that other GABAergic ligands also have an effect on the EEG, these studies can be extended by using subunit selective ligands, such as barbiturates (selective for α_6), zolpidem (selective for α_1), loreclezole (selective for β_2 and β_3), tracazolate (selective for β_3) or furosemide (selective for α_4 and α_6).[34,37,38] This will provide *in vivo* information on the time course of alterations in receptor subtypes and their functionality. For zolpidem it has been shown already that the total amplitude of the β-frequency of the EEG is a useful measure to quantify its efficacy.[39]

For quantification of the efficacy of GABAergic acting compounds, usually the alteration in the total amplitude of the β-frequency band, defined as 11.5–30 Hz, of the EEG is used.[40–43] However, figure 9.2 indicates that there are also ligand-specific changes in other frequencies. This figure shows the spectrograms of EEG recorded after administration of midazolam, tiagabine and alphaxalone to control animals, indicating that alphaxalone has a clear effect on the frequencies of 8–10 Hz. Tiagabine, in contrast, most prominently increases the amplitude at ~5 Hz, an effect which is also vaguely visible after administration of midazolam. These results suggest that a more detailed analysis of the pharmaco-EEG before and after epilepsy induction might be useful to investigate alterations in the functionality of the $GABA_A$ receptor subtypes.

To get more insight into the expression of subunits of the $GABA_A$ receptor *in vivo*, PET scanning is an interesting technique. Preferably, subunit selective compounds which are labelled with a positron emitter, such as ^{11}C or ^{18}F, should be used as ligand. Development of such ligands, however, is very cost and time consuming. As an alternative, competition experiments in which binding of [^{11}C]labelled flumazenil is inhibited by subunit selective ligands could be performed. This would provide *in vivo* information about the time course of changes in subunit composition of the $GABA_A$ receptor.

Another feature of PET experiments with subunit selective ligands, is the possibility to examine the regional distribution of receptor subtypes, although this is limited by the spatial resolution of the PET scanner. There is accumulating evidence that individual $GABA_A$ receptor subtypes are associated with distinct neuronal structures and subcellular distributions, and that their differential activation is correlated with distinct pharmacological and behavioral phenotypes.[44] This implies that alterations in the regional distribution of (subtypes of) the $GABA_A$ receptor and even changes within certain neuronal circuitries are important in the study of epilepsy and pharmacoresistance.

The importance of taking into account the subunit expression at certain cell types within neuronal circuitries is illustrated by the different action of benzodiazepines as compared to GABA itself on the duration of spike wave discharges in animal models of absence seizures. Administration of GABA transaminase inhibitors or GABA reuptake inhibitors, resulting in higher levels of GABA, causes an exacerbation of the duration

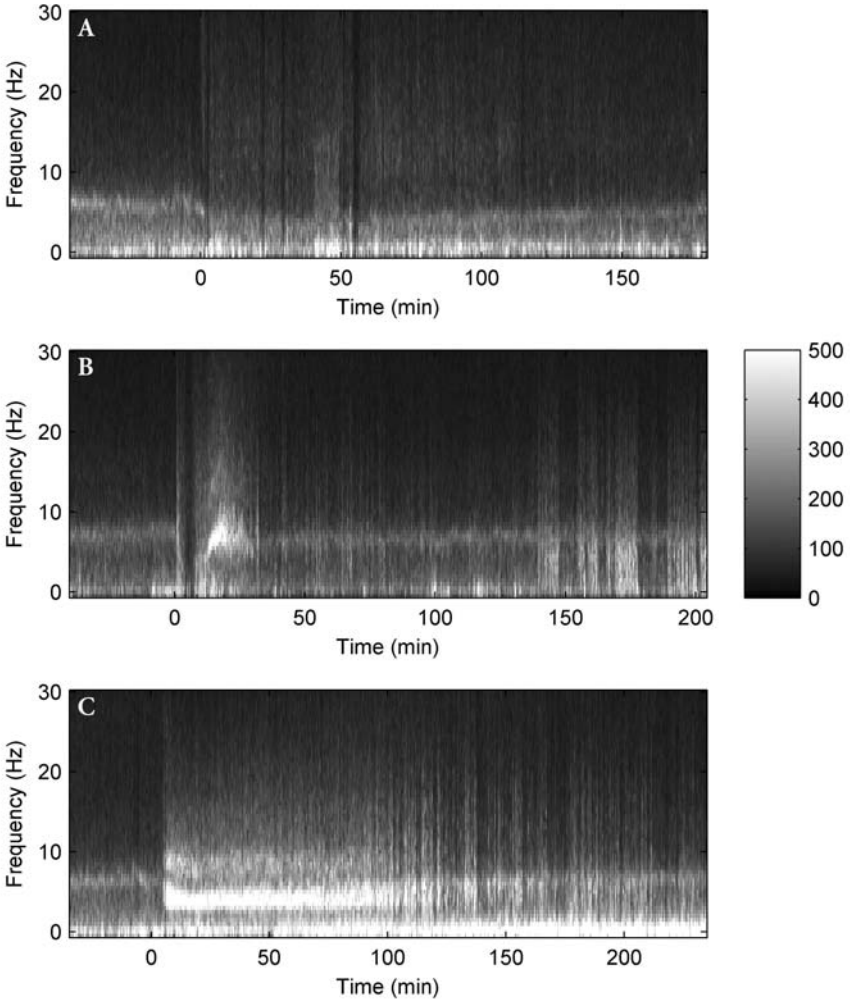

Figure 9.2: Differential effect of GABAergic ligands on the EEG. Shown are spectrograms of EEG obtained after intravenous administration of midazolam (10 mg/kg in 2 minutes; panel A), alphaxalone (10 mg/kg in 5 minutes; panel B) and tiagabine (10 mg/kg in 10 minutes; panel C), in control rats. At time = 0 the infusion was started. The greyness is a measure for the amplitude of the frequencies, as indicated by the colourbar. Frequencies from 0–10 Hz only are shown, because EEG was band-pass filtered at 30 Hz (see Chapters 7 and 8 for technical details).

of the spike wave discharges.[45] The benzodiazepine clonazepam, however, which is an allosteric modulator of the $GABA_A$ receptor, has a therapeutic effect in the treatment of absence seizures.[45,46] The reason for this dichotomy is that clonazepam enhances $GABA_A$ receptor mediated inhibition only within certain cells of the thalamocortical circuit, whereas GABA itself enhances GABAergic inhibition in a more general manner within all cells of the circuit, which leads to the observed antitherapeutic effect.[45,46]

Summarising, the studies in this thesis have shown that $GABA_A$ receptor expression can be quantified using PET studies with [^{11}C]labelled flumazenil. Extending the protocol with competition experiments using subunit selective ligands is expected to give insight into amount and regional distribution of specific subtypes of the receptor. Moreover, subunit specific changes in receptor functionality were found with PK-PD experiments using compounds that are selective for specific subunits. So far, the total amplitude of the β-frequency band of the EEG was used to measure the effect. When analysing the EEG in more detail, also ligand specific EEG changes can be observed, suggesting that analysis of pharmaco-EEG can help to investigate the pharmacology of $GABA_A$ receptor subtypes.

9.4.3 Extrapolation to clinical situation

Animal studies are very useful for investigating mechanisms that may underlie epilepsy and pharmacoresistance. Ultimately, however, results obtained in animals should be translated to the clinical situation. In that respect, it would be important that the same tools for quantification of epileptogenesis and pharmacoresistance development can be used in both animal models and people with epilepsy. All techniques used in this thesis fulfil this condition. It was shown that analysis of continuously recorded EEG by quantifying interhemispheric synchronisation of EEG events is a promising method to measure disease progression (Chapter 4). Since EEG recordings were derived from the cortex of the animals, it is expected that the results obtained with this method can easily be extrapolated to routinely measured EEGs in the clinic.

Quantification of the $GABA_A$ receptor *in vivo* using PET and [^{11}C]labelled flumazenil has been widely used in the clinic. In Chapter 5 a novel method is presented to obtain independent estimates of B_{max} and K_D using a single PET experiment, by making use of the complete range of receptor occupancies. This was achieved by administration of a saturating dose and taking advantage of the short half-life of flumazenil of the rat ($t_{1/2}$ = 8.3 minutes[25]). The latter property made it possible to cover the entire range of receptor occupancies between 100 and 0% during a scan of 30 minutes. Since flumazenil has no pharmacological and toxicological effects, the proposed method could, at least in theory, also be used in humans. However, it should be noted that the physiological half-life of flumazenil in human is significantly longer (49 to 58 minutes[47]), implying that it would take at least 3 hours to clear a saturating dose from the human body. Obviously, for use in humans some adjustments to the protocol are required.

Finally, the functionality of the $GABA_A$ receptor was investigated by performing PK-PD experiments with GABAergic ligands and cortical EEG as pharmacodynamic

endpoint. A few examples of studies on the exposure response relationship of such ligands have been published (see for instance[48–50]), illustrating the possibility to perform these studies in a clinical situation. An advantage of measuring EEG in humans is that many more electrodes can be used than in animals. This provides the opportunity to investigate the EEG response to GABAergic ligand administration in more detail.

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