Chapter II

General introduction: Disease progression and pharmacoresistance in epilepsy

2.1 Introduction

Epilepsy has been known since ancient times.\(^1,2\) Around 40 AD Celsus described many remedies against this “falling sickness”, such as drinking hot blood from the cut throat of a gladiator, letting a little blood from both legs near the ankle, or incising the back of the scalp.\(^2\) Currently, much more is known about epilepsy and a wide range of antiepileptic drugs is available. The conclusion of Celsus, however, that “if the disease has not been brought to an end by the foregoing measures, it is probable that it will be lifelong” still remains true.\(^2\) As it is still unknown why it is not possible to control seizures in all people with epilepsy by use of existing antiepileptic drugs, both the development of epilepsy and pharmacoresistance are important topics of research.

In this chapter some background on epilepsy in relation to the development of pharmacoresistance is provided, with specific emphasis on the role of the GABA\(_A\) receptor. Furthermore, the use of animal models, biomarkers and population modelling in the study of epilepsy and pharmacoresistance is discussed.

2.2 Epilepsy and pharmacoresistance

Epilepsy is a common neurological disorder with an estimated prevalence in Europe of 4.3–7.8 per 1000,\(^3\) and as high as 16–20 per 1000 in Africa.\(^4\) Clinically, epilepsy is characterised by recurrent spontaneous seizures. An epileptic seizure is an acute and transient event, which is manifested as a brief change in behaviour caused by the disordered, synchronous and rhythmic firing of populations of neurons in the central nervous system.\(^5\) A wide diversity of epilepsy syndromes have been described, based upon several characteristics, such as symptoms, seizure types and electroencephalographic patterns.\(^5,6\)

A well known issue in epilepsy is the occurrence of pharmacoresistance. The importance of this issue is illustrated by the fact that about 20–30% of all people with epilepsy have poorly controlled seizures, or their seizures are refractory to drug treatment.\(^7,8\) Not all forms of epilepsy have the same rate of pharmacoresistance. Generalised epilepsies, for instance, are only infrequently associated with resistance to antiepileptic drugs. Examples of epilepsy syndromes with a high incidence of therapy resistance are the Lennox-Gastaut syndrome and mesial temporal lobe epilepsy (mTLE\(^9,8\)). The Lennox-Gastaut syndrome has a prevalence of only 2% of all people with
epilepsy, whereas mTLE is an epilepsy syndrome affecting many people with epilepsy: a study at a large epilepsy referral centre in Paris found that about 41% of their patients had a diagnosis of mTLE. Because of the high risk of therapy resistance in this latter form of epilepsy, together with its relatively high prevalence, most studies on pharmacoresistance in epilepsy have involved mTLE. Several animal models for mTLE are available, which significantly facilitates studying both epilepsy and pharmacoresistance.

2.2.1 Mesial temporal lobe epilepsy
Mesial temporal lobe epilepsy has a characteristic clinical presentation, which seems progressive in nature. As reviewed by Engel, patients often have a history of complicated febrile seizures or other initial precipitating injuries, such as head trauma or intracerebral infections, within the first four or five years of life. There is also an increased prevalence of a family history of epilepsy. In the latter half of the first decade of life habitual seizures start to occur, which in most cases can be brought under control by pharmacotherapy. Subsequently, the seizures often remit for several years until adolescence or early adulthood. After this latent period habitual and complex partial seizures develop, which are mostly insensitive to medication. At this stage, about 60% of the patients become pharmacoresistant.

A habitual seizure typically starts with an aura, which usually lasts for several seconds. These auras might be followed by complex partial seizures. These seizures typically begin with motor arrest and staring, followed by oromotor automatisms, such as lip-smacking and chewing, and other purposeless movements. The postictal phase usually includes confusion, recent-memory deficit and amnesia for the event.

Neuropathologically, mTLE is associated with extensive neuronal cell loss in the hippocampus, or hippocampal sclerosis, reactive gliosis and mossy fibre sprouting and reorganisation in the dentate gyrus. It is unclear whether this abnormality is the cause of the epileptic condition or the consequence of repeated seizures and the question has been an important topic in the debate about temporal lobe epilepsy.

An important diagnostic hallmark of mTLE is interictal anterior temporal spike-wave discharges on the EEG. Characteristically, auras are not associated with ictal EEG changes, but a typical ictal EEG onset pattern of five to seven Hz rhythmic activity is seen at the onset of the complex partial seizure. Apart from EEG, several neuroimaging techniques can be used to confirm the diagnosis. For instance, PET with \(^{18}\)F-labelled fluorodeoxyglucose (FDG) can detect interictal hypometabolism, which is an indication of hippocampal sclerosis and other mesial temporal lesions. Another way of showing hippocampal sclerosis is by means of MRI scanning.

2.2.2 Pharmacoresistance
An exact, universally acceptable definition of pharmacoresistance is difficult to provide. Strictly speaking, pharmacoresistance would mean that it is not possible to reduce seizure frequency with any antiepileptic drug at any concentration, which would imply
that poor or moderate reduction in seizure frequency should not be considered as pharmacoresistance. Poor seizure control, however, is clearly unacceptable from a clinical point of view. Total absence of seizures is the aim and if this cannot be achieved, seizure control may be considered satisfactory only if seizures occur very rarely and without appreciable side effects. A good example of a definition, specifying seizure frequency, the number of tried antiepileptic drugs and the period of observation, is given by Berg et al.\textsuperscript{16} They defined pharmacoresistance as “failure, for lack of seizure control, of more than 2 first line antiepileptic drugs, with an average of more than 1 seizure per month for 18 months and no more than 3 consecutive months seizure-free during that interval. Drugs had to be pushed to maximum tolerated doses”.

The exact specifications, however, remain a matter of debate\textsuperscript{17,18} and different definitions may be needed depending on the objective of a study.\textsuperscript{18} For example, using the definition by Berg et al, it is not possible to quantify progression of pharmacoresistance, to take into account the natural history of disease progression, or to quantify the degree of improvement by drug treatment. In this respect, the studies by Arts et al revealed a more refined picture.\textsuperscript{19} They followed 453 children with newly diagnosed epilepsy for 5 years. At two timepoints within this period, at 2 and 5 years after inclusion, they recorded the duration of the seizure free period during the preceding time. By comparing these values at 2 and 5 years after inclusion, improvement and deterioration could be determined, in addition to complete absence of seizures and total unresponsiveness.

In experimental studies in animals, however, it is impractical to test several antiepileptic drugs for prolonged periods of time to establish whether or not an animal is or becomes pharmacoresistant. For this reason, it is proposed to define pharmacoresistance as “the inability of an antiepileptic drug to achieve more than 50% seizure reduction at concentrations that do not cause unacceptable adverse effects”. The arbitrarily chosen cut-off point of 50% allows further refinement, for instance by making a distinction between total and partial pharmacoresistance (e.g., 0–50 and 50–90% reduction, respectively) or even between severe, moderate and slight pharmacoresistance (e.g., 0–30, 30–60, 60–90% reduction, respectively).

This definition has several practical advantages. Firstly, an upper limit is set for drug concentrations that are considered unacceptable, to exclude concentrations that cause toxic effects. Furthermore, in this way pharmacoresistance is defined for individual drugs and for a single time point, i.e., the time at which the drug is tested, allowing characterisation of the time course of development of pharmacoresistance.

This is an important advantage when studying mechanisms underlying drug resistance, which are only poorly understood at present. It might be related to the repeated administration of antiepileptic drugs, leading to tolerance development, as extensively reviewed by Löscher and Schmidt.\textsuperscript{20} On the other hand, development and progression of epilepsy themselves might induce changes which result in the development of pharmacoresistance. As the investigations in this thesis focus on the relationship
between epileptogenesis and the development of pharmacoresistance, the remainder of this paragraph will focus on the contribution of latter mechanism.

2.2.3 Mechanisms underlying development of pharmacoresistance
With regard to epilepsy related mechanisms underlying pharmacoresistance, two main concepts have been proposed: (a) the multidrug-transporter hypothesis, suggesting that over expression of multidrug-transporters at the blood-brain barrier limits uptake of antiepileptic drugs in the brain and (b) the drug-target hypothesis, which suggests that alterations of drug targets within the brain lead to reduced or absent pharmacosensitivity.21,22

Within the brain, several multidrug-transporters are located at the apical, or luminal, membrane of the endothelial cells that form the blood-brain barrier. These transporters reduce brain penetration and increase brain extrusion of many drugs.8 This implies that upregulation of these transporters will result in lower drug levels in the brain, which in turn may cause pharmacoresistance. Enhanced expression of several multidrug-transporters has been shown both in epileptogenic brain tissue resected from patients with intractable epilepsy and in rodent epilepsy models (for review, see8,23). This upregulation might be caused by genetic factors, as it is known that certain polymorphisms in the MDR1 gene that encodes Pgp in humans are associated with increased expression and functionality of this transporter.23 On the other hand, it might be induced by an epilepsy related factor, such as uncontrolled seizures. In response to prolonged seizure activity, the blood-brain barrier is opened, which might cause an upregulation of multidrug-transporters, thereby functioning as a defence mechanism.23

These findings suggest that co-administration of an antiepileptic drug and a Pgp inhibitor may provide a novel treatment strategy for patients with intractable epilepsy.23,24 There is, however, also substantial evidence that the drug targets themselves are altered.25 Thus, even when obtaining high concentrations of antiepileptic drugs at the site of effect, these drugs might be ineffective, due to altered receptor function. The most important targets of antiepileptic drugs are voltage-gated cation channels, like the Na⁺ channels and Ca²⁺ channels, or the GABAergic system.26 A complete and long-lasting loss of the effects of carbamazepine on Na⁺ channels was found in the pilocarpine model of epilepsy and in people with carbamazepine-resistant temporal lobe epilepsy.22 Although the importance of changes in the Ca²⁺ channels for pharmacoresistance to lamotrigine has not been directly addressed, it is conceivable that these changes could underlie a loss of the effect of lamotrigine.22

Alterations in the GABAergic system have also been extensively studied. For instance, in people with epilepsy and in animal models a decrease in GABA_A receptor binding has been shown, leading to reduced efficacy of GABAergic antiepileptic drugs. The GABAergic system is interesting for more than one reason. Apart from being a target for various antiepileptic drugs, it is also an important mechanism for controlling excessive excitatory activation.27,26
2.3 GABAergic inhibition in epilepsy

The \( \gamma \)-aminobutyric acid (GABA) system plays an important role in the central regulation of somatic and mental functions, as GABAergic inhibition controls spike-timing and oscillatory network activity.\(^{27}\) Impairment of this inhibition may lead to excessive activation of excitatory neuronal circuits, as is the case in epilepsy. Therefore, alterations in the GABAergic system have been the focus of recent research.\(^{28}\) These alterations might be either presynaptic, including a decrease in GABA synthesis or release and an increase in reuptake, or postsynaptic, including alterations in GABA\(_A\) receptors or events downstream from receptor activation.\(^{28}\) Knowledge about alterations in the GABAergic system in epilepsy may lead to better understanding of the disease, which would make it possible to develop adequate treatment, and would also lead to understanding of the development of pharmacoresistance against GABAergic acting antiepileptic drugs.

2.3.1 Presynaptic processes

GABA is presynaptically formed through decarboxylation of glutamic acid catalysed by glutamic acid decarboxylase (GAD).\(^{29}\) The release of GABA into the synaptic cleft primarily is a vesicular process, which is controlled by several presynaptic autoreceptors, such as specific kainate receptors, \( \alpha_{1A} \) adrenoceptors and GABA\(_B\) receptors.\(^{30,31}\) Nonvesicular GABA release occurs secondary to depolarisation of the cell membrane and sodium influx, and is a Ca\(^{2+}\) independent process.\(^{32}\) Following its release in the synaptic cleft, GABA can either activate the postsynaptic GABA\(_A\) receptor, or can be taken up by the presynaptic nerve terminal or glial cells via reuptake transporters.\(^{30}\) Finally, GABA is catabolised by GABA transaminase to succinic semialdehyde.\(^{32}\)

A decrease in GABA synthesis or release, or an increase in GABA reuptake and catabolism will result in lower GABA concentrations in the synaptic cleft, and thus to impaired GABAergic inhibition. Several studies on GAD expression in human TLE and different animal models of epilepsy have shown increased levels of GAD mRNA and protein expression in the hippocampus.\(^{33–35}\) This suggests that GABA synthesis is not impaired, but might even be increased, pointing to a possible protective mechanism to limit seizure activity.\(^{33}\)

The role of the GABA\(_B\) receptor in epilepsy has been investigated in several studies. For instance, knocking out specific subunits of the GABA\(_B\) receptor in mice resulted in spontaneous generation of (primarily clonic) epileptic seizures.\(^{36}\) Straessle \textit{et al} showed that 24 hours after the start of kainic acid-induced status epilepticus in mice, the GABA\(_B\) receptor immunoreactivity was profoundly reduced in the hippocampus.\(^{37}\) Interestingly, at later stages after status epilepticus they found increased GABA\(_B\) receptors density in dentate gyrus granule cells and specific subtypes of interneurons.\(^{37}\) The observed rapid kainic acid-induced loss of GABA\(_B\) receptors might contribute to epileptogenesis because of a reduction in both presynaptic control of transmitter release and postsynaptic inhibition. In turn, the long-term increase in GABA\(_B\) receptors in granule cells and
specific subtypes of interneurons may represent a compensatory response to recurrent seizures.37

Taken together, there is not much evidence that impairment of the synthesis and release of GABA are major causes of decreased GABAergic inhibition, leading to epileptic seizures. Neither is the role of alterations in reuptake of GABA in epilepsy clear, as some studies report a downregulation of reuptake transporters, whereas others report increased levels.38 As both specific inhibition of GABA reuptake by tiagabine, and vigabatrin induced reduction of GABA catabolism result in less epileptic activity, it can be concluded that GABAergic inhibition does play a role in epilepsy.32,39,40 This suggests a contribution of impairment of postsynaptic processes, including alterations in postsynaptic receptor properties and events downstream from receptor activation.

2.3.2 Postsynaptic processes

Postsynaptically, GABA binds to the GABA<sub>A</sub> receptor, which is a pentameric chloride (Cl<sup>−</sup>) channel composed of various combinations of several classes of subunits (α<sub>1</sub> to α<sub>6</sub>, β<sub>1</sub> to β<sub>3</sub>, γ<sub>1</sub> to γ<sub>3</sub>, δ, ε, π, θ, and ρ<sub>1</sub> to ρ<sub>3</sub>). The subunit composition of the GABA<sub>A</sub> receptor determines its pharmacological and electrophysiological properties.43 Upon GABA binding, the Cl<sup>−</sup> channel is opened, allowing a rapid flux of Cl<sup>−</sup>. As most neurons maintain a low intracellular Cl<sup>−</sup> concentration, this flux is predominantly inwards, resulting in hyperpolarisation and shunting of excitatory synaptic input. This elicits inhibitory postsynaptic potentials, thus decreasing the probability of generating an action potential.42 The number and composition of GABA<sub>A</sub> receptors available to respond to released GABA is regulated by means of so called receptor trafficking.44,45 Receptor trafficking involves intracellular movement of receptors from sites of synthesis to the plasma membrane, where they function, and then to sites of degradation.44

There is substantial evidence for alterations of GABA<sub>A</sub> receptors in epilepsy, resulting in decreased GABAergic effects.46 In both human and animal studies a decrease in benzodiazepine binding has been shown. For instance, ex vivo studies with the [<sup>3</sup>H]labelled benzodiazepine antagonist flumazenil in brain tissue of patients showed that the binding of flumazenil was decreased in different hippocampal areas.47 The same results were found in vivo in a PET-study with [<sup>11</sup>C]flumazenil in people with TLE.48 In both studies the decrease in GABA<sub>A</sub> receptor binding was more pronounced than the neuronal cell loss, suggesting not only neurodegeneration of GABAergic neurons, but also disappearance of GABA<sub>A</sub> receptors.48 Experimental studies in post-status epilepticus models of mTLE, such as the pilocarpine or kainate models, have also shown that epileptogenesis is associated with profound changes in GABA<sub>A</sub> receptors. In the kainic acid induced post-status epilepticus model binding of [<sup>3</sup>H]flunitrazepam or expression of GABA<sub>A</sub> receptor mRNA were altered in different brain regions at 12 or 24 hours after onset of status epilepticus.49,50

The exact mechanisms underlying the observed decreases in GABA<sub>A</sub> receptor binding and expression are still uncertain. Interesting in this respect is a study performed by
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Naylor et al. At 1 hour after onset of status epilepticus in the rat they reported a 50% decrease in the number of physiologically active GABA_\text{A} receptors in dentate granule cells, and a commensurate increase in receptor subunits in the cytoplasm. This indicates that the receptor is internalised during status epilepticus. Moreover, they showed that the loss of physiologically active receptors could be mimicked by a 20 minute exposure of brain slices to GABA. This suggests that an increase in GABA release as result of seizure activity contributes to the observed GABA_\text{A} receptor internalisation.

Another often reported observation is the rapid and extensive change in subunit expression of the GABA_\text{A} receptor during epilepsy and status epilepticus. As the pharmacological and electrophysiological properties of the GABA_\text{A} receptor are highly dependent on the subunit composition of the receptor, these changes may lead to altered functionality. Although the subunit composition of the GABA_\text{A} receptor has relatively little influence on activation of the receptor by GABA, this changed functionality may have significant impact on the efficacy of GABAergic antiepileptic drugs.

2.3.3 GABA_\text{A} receptor subtypes

As mentioned, a total of 6\(\alpha\), 3\(\beta\), 3\(\gamma\), 1\(\delta\), 1\(\epsilon\), 1\(\pi\), and 3\(\rho\) subunits of GABA_\text{A} receptors have been identified. If all these subunits could randomly co-assemble with each other, more than 150,000 GABA_\text{A} receptor subtypes with distinct subunit composition and arrangement could theoretically be formed. However, due to restrictions imposed during assembly of GABA_\text{A} receptors, not all of them are actually formed in the brain. The most common subunit composition has been determined to contain \(\alpha\), \(\beta\), and \(\gamma\) subunits. The \(\delta\), \(\epsilon\), and \(\pi\) subunits seem to be able to replace the \(\gamma\) subunit in GABA_\text{A} receptors, whereas the \(\theta\) subunit might be able to replace a \(\beta\) subunit in these receptors. The availability of a \(\gamma_2\) subunit is crucial for modulation by benzodiazepines. A loss or replacement of this subunit would render the receptor insensitive to benzodiazepines. It is even important which \(\alpha\) subunit is present, as the presence of \(\alpha_4\) causes inefficacy of midazolam. Loreclezole sensitivity is determined by the presence of a \(\beta_2\) or \(\beta_3\) subunit, whereas neurosteroids are specifically sensitive to GABA_\text{A} receptors with a \(\delta\) subunit.

Changes in subunit composition of the GABA_\text{A} receptor as result of epileptogenesis have been explored extensively, showing great variety in subunit changes. For instance, immediately after induction of status epilepticus with kainic acid in rats, a decrease in \(\alpha_2\), \(\alpha_3\), \(\alpha_5\), \(\gamma_2\) and \(\delta\) subunits was observed in the dentate gyrus of the hippocampus, whereas \(\alpha_1\) and \(\alpha_4\) subunits were increased in the same area. Another study showed that during the latent period, \(\alpha_3\), \(\alpha_4\) and \(\delta\) subunits were increased in single dentate granule cells after induction of status epilepticus with pilocarpine, whereas the quantity of \(\gamma_2\) subunits was unaltered. During the period of spontaneous seizures, some of these changes persisted, whereas others reversed. Thus various alterations in subunits after induction of status epilepticus have been reported, but these alterations vary among different cell types or brain areas, and over time.

In summary, much is known about GABAergic inhibition, and alterations and/or im-
pairment in GABAergic inhibition in epilepsy. These changes appear to be predominantly postsynaptic, and more specifically, related to the GABA<sub>A</sub> receptor. However, the exact contribution of these alterations to development of epilepsy and pharmacoresistance has not yet been quantified. It is important to study this causality, as it might provide a lead with respect to preventing epileptogenesis and the development of pharmacoresistance by reversing these changes.

2.4 Animal models

Development of epilepsy and pharmacoresistance involves many mechanisms and factors: it would be better to study these mechanisms in humans. Performing studies in humans, however, presents many problems, such as ethical issues, poor reproducibility and confounding effects of medication. As an alternative, human material obtained from surgery could be used. This has, however, some major drawbacks, as surgery is performed only in an advanced stage of the disorder. This makes it impossible to study the development of the epileptogenic process and pharmacoresistance. Moreover, only isolated processes involved in the disease can be studied, whereas the relative contribution of these can only be investigated in complete subjects. Therefore, most research into disease development is performed in animal models.

For epilepsy research, various animal models are available, each with their specific characteristics (for a complete account, see Pitkänen et al<sup>57</sup>). Globally, these models are categorised into models of seizures and those of epilepsy.<sup>58</sup> Examples of seizure, or acute, models are the cortical stimulation model and the maximal electroshock model. The amygdala kindling model is also an acute model, as most animals do not develop spontaneous epilepsy. The models of epilepsy, or chronic models, can be subdivided into models of genetic (idiopathic) epilepsy and models of acquired (symptomatic) epilepsy.<sup>59</sup> The first category includes both animals with spontaneous mutations, such as the Genetic Absence Epilepsy Rat from Strasbourg (GAERS), and animals, usually mice, with induced mutations, resulting in epileptic symptoms and behaviour.<sup>59</sup> In animals of the acquired or symptomatic epilepsy models, status epilepticus is induced by electrical or chemical methods in previously non-epileptic rats, which results in the development of spontaneous seizures.<sup>59,11</sup>

The choice of the model to be used depends on the research question. The investigations in this thesis focus on the relationship between epileptogenesis and the development of pharmacoresistance, with strong emphasis on alterations in the drug target. A useful animal model should therefore meet the following criteria, partly based on the selection criteria proposed by Sarkisian<sup>58</sup> and Löscher. Firstly, the disease should be progressive. Secondly, seizures should be similar in their clinical phenomenology to those occurring in human mTLE.<sup>60</sup> Thirdly, seizures should be associated with paroxysmal activity in the EEG.<sup>60</sup> Fourthly, the aetiology should be similar to allow extrapolation of possible alterations in drug target.<sup>58</sup>

The animal model that meets these four criteria is the post-status epilepticus model:
after a primary insult, the induced status epilepticus, and a subsequent latent period, spontaneous progressive seizures develop. As will be discussed in more detail below, the other criteria are also met in this animal model. However, a practical drawback of using these animals is the inter-animal variability in rate and occurrence of spontaneous seizure development. Therefore, in experiments which require a more controllable animal model with low inter-animal variation, the amygdala kindling model is a good alternative.

2.4.1 Amygdala kindling
Since its initial description by Goddard in 1967,61 the kindling model has been used extensively as an animal model of epilepsy.60,11 Kindling is an apparently simple phenomenon in which repeated mild electric stimulations with a constant current are applied to the rat brain via an implanted electrode. This results in an epileptic response, which is progressive over the course of kindling. After about 15 stimulation sessions, the rat reliably displays class V seizures, according to Racine's scale.62 At this stage, the animal is said to be fully kindled, a state which persists for months to years.11 In a fully kindled animal, electric stimulation will evoke a class V seizure. Only very rarely, however, does the animal show spontaneous seizures. Development of spontaneous seizures can be induced by continuing the kindling sessions to about 250, demonstrating that kindling has resulted in epileptogenesis.62,60

Advantages of the kindling model include the possibility to activate precisely target brain sites, the low inter-animal variability, and the controlled induction of a seizure. Unless a very large number of kindling stimulations are applied, however, the animals display no disease progression.11 Therefore, this animal model is very useful for studying the effects of seizures themselves. For investigating the causes and underlying mechanisms of epileptogenesis and development of pharmacoresistance, progressive animal models are preferable.

2.4.2 Post-status epilepticus animal model
Spontaneous seizures occur in the animals of the kindling model only after a large number of kindling stimulations. A faster way to develop spontaneous seizures is the induction of status epilepticus, either chemically or electrically. Electrical stimulation of specific brain regions or administration of certain chemicals, such as kainic acid or pilocarpine, causes excessive glutamate receptor activation. This results in a continuous state of GABAergic disinhibition, leading to the development of status epilepticus.11 This continuous depolarisation can be excitotoxic, thus damaging a wide range of cells, including hippocampal pyramidal neurons, dentate hilar neurons, and piriform cortical neurons. During the subsequent latent period, in which no seizures occur, numerous transcription factors and genes linked to cytoskeletal and synaptic reorganisation are upregulated, resulting in synaptic plasticity and reorganisation in glutamate and GABA systems.11 It is hypothesised that this reorganisation leads to progressive spontaneous seizures, which start to develop after a silent period of weeks to months.
This pattern of development of experimental epilepsy in the post-status epilepticus models closely mimics the human situation for mTLE, where epilepsy generally develops some time after an initial insult. In addition, the morphological changes that occur in the hippocampus following status epilepticus are often quite similar to those seen in human mTLE, although the damage in the animal model can be more severe and widespread.59 Concerning seizure semiology, the post-status epilepticus models also closely resemble human mTLE. As reviewed by Sperk,63 systemic administration of kainic acid produces various motor signs including convulsive seizures. The seizures do progress over time, starting with staring, head nodding, facial twitches and wet dog shakes, and progressing into severe limbic motor seizures involving the whole body.63 Moreover, observed behavioural changes strikingly correlate with electrographic activity, as is the case in human mTLE.63

A potential practical limitation of the model is the fact that the response to status epilepticus is variable. Consequently, expression of the spontaneous seizures can be unpredictable, and in addition, there is more inter-animal variation, might be seen as a practical limitation of the model.59 On the other hand, the inherent variation provides an opportunity to correlate clinical outcome measures to some aetiological or pathological biomarkers.

### 2.5 Biomarkers for disease progression in epilepsy

Biomarkers are useful tools for a quantitative description of disease progression and development of pharmacoresistance. The Biomarkers Definitions Working Group has defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”.64 Within the context of pharmacokinetic-pharmacodynamic (PK-PD) modelling Danhof et al proposed a seven point scale for the classification of biomarkers, based on the location of the biomarker in the chain of events from underlying genotype or phenotype to clinical scales.65 The subsequent biomarker types are:

- type 0: genotype or phenotype
- type 1: concentration of a drug and/or drug metabolite
- type 2: target occupancy
- type 3: quantification of target activation
- type 4: physiological measures in the integral biological system
- type 5: parameters that characterise disease processes quantitatively
- type 6: clinical scales
As both disease progression and pharmacoresistance are chronic and progressive, it is important that measurements can be repeated, indicating the importance of non-invasive and repeatable biomarkers. Moreover, one of the hallmarks of the development of epilepsy is a silent period, in which no seizures occur. As it is hypothesised that alterations leading to pharmacoresistance originate during this silent period, this period must be quantified as well.

In the present investigations, a number of different biomarkers, meeting the criteria as described above, were used. Firstly, PK-PD experiments with different GABAergic ligands were performed, using EEG alterations as effect measure or PD endpoint. As the drug concentrations were also measured, these experiments give insight into type 1 (concentration of drug and/or metabolite) and type 3 (quantification of target activation) biomarkers. Secondly, positron emission tomography (PET) with the $[^{11}C]$labelled GABA$_A$ receptor antagonist flumazenil as a ligand resulted in quantification of GABA$_A$ receptor binding, expressed as $B_{\text{max}}$ and $K_D$, and transport of flumazenil across the blood-brain barrier. Thus, as this provides insight into target occupancy, it can be used as a type 2 biomarker. Thirdly, quantification of disease processes was studied using electric stimulation of the cortex and cortical EEG measurement. Finally, evaluation of behavioural responses to electric stimulation of the cortex was investigated as a clinical scale, or type 6 biomarker.

### 2.5.1 Electroencephalography

An electroencephalogram (EEG) is a record of the electric activity of the brain, usually obtained from the scalp. After amplification, the signal is usually saved in graphic or digital format. During past decades, the EEG has been an important tool for obtaining knowledge about the normal functioning brain, and also about dysfunctioning of the brain as a result of various neural diseases. As EEG is a non-invasive and quantitative method which can be used repeatedly, it also offers interesting opportunities to quantify disease progression in neuropathological disorders. Moreover, as a number of centrally acting drugs affect the EEG in a drug specific manner, alterations in the EEG have been used as a tool for quantifying drug effects.

When using EEG for quantification of disease progression in epilepsy, a few issues should be considered. Firstly, as it concerns continuous and longitudinal measurements, a massive amount of data will be generated. Although rapid advances in computer technology and data saving capacity makes this issue less important, it is worth critically considering the amount of data needed. Data reduction can effectively be achieved by reducing sampling frequency. However, the sampling frequency should be high enough to ensure that rapid events of interest are recorded accurately. In theory, the sampling frequency should be greater than twice the maximum frequency of interest.

Apart from this technical consideration, it is important to note from which part of the brain the EEG is measured. In human studies usually the EEG is measured from the scalp, but in animal studies it is possible to measure electrical activity in specific brain areas.
regions. For that purpose, in preclinical epilepsy research, often the EEG is measured in the amygdala or hippocampus, which are important sources of epileptic discharges. Due to its invasive nature, however, deep-brain EEG recordings are used only very sporadically in humans. To facilitate translational research between animal models and the clinic, cortical EEG recordings are preferred. Moreover, as motor behaviour originates in the cortex, this provides a rationale for correlating cortical EEG recordings with motor events, such as epileptic seizures.

Finally, the method for analysing EEG data should be chosen. Despite all advances in analytical methods for detecting epileptic seizures, the most reliable method still is visual EEG analysis by experienced readers. However, this method is very time consuming, may not be sensitive to gradual trends in the EEG such as changes in variability of frequency content, and is highly dependent on the availability of experienced personnel. Therefore, several computerised methods have been developed for quantification and visualisation of certain aspects of the EEG. Most of these methods are based on the frequency domain methods using Fourier analysis, amplitude-integrated EEG, or EEG bispectrum. A review on currently available techniques for display and analysis of continuous EEG data can be found elsewhere.

The EEG is an attractive measure for quantification of the effect of CNS active drugs, as it can be measured continuously, and is sensitive and reproducible. For quantification of GABAergic acting compounds, the alteration in the total amplitude of the β-frequency band of the EEG is commonly used. Studies by Mandema et al have shown that in vivo efficacy and potency, derived from the β-activity of the EEG, correlate well with in vitro efficacy and affinity of a whole spectrum of benzodiazepines, from full agonists to inverse agonists. Moreover, it has been shown that both potency and maximal EEG effect for different benzodiazepines closely correlate with their ability to suppress seizure activity. Cleton et al showed that the GABA reuptake blocker tiagabine increases the β-activity in a concentration-dependent way to a similar level as the benzodiazepines. In addition, they reported a similar difference between in vivo potencies of R- and S-tiagabine as compared to their difference in in vitro binding affinity to the GABA reuptake transporter, supporting the hypothesis that the increase in β-activity of the EEG is a quantitative measure of the effects of tiagabine on the GABAergic system. As a last example, administration of neurosteroids also increases the total amplitude of the β-frequency of the EEG, but to a much higher level and in a biphasic manner. Analysis of a range of different neurosteroids showed that these compounds exhibit the same intrinsic efficacy, but that they differ in potency. These differences in in vivo potency correlate well with the in vitro IC50 obtained in a [35S]TBPS binding assay.

In conclusion, the EEG is a valuable biomarker, as it represents a non-invasive and repeatable measurement that can be applied both in experimental animals and in humans. It is causally related both to pathology of epilepsy, thus signifying its usefulness in quantifying epilepsy progression, and to GABAergic effects induced by GABAergic acting compounds. This latter property makes it a valuable technique to study
differences in GABAergic effects during the process of epileptogenesis and development of pharmacoresistance.

2.5.2 Positron Emission Tomography

Positron emission tomography (PET) is a powerful in vivo technique to non-invasively quantify the distribution of a compound, labelled with a positron emitting radionuclide, over time. It makes use of radionuclides that decay by emission of a positron. This positron travels at most a few millimetres in tissue before combining with an electron. These two particles then annihilate, resulting in the simultaneous emission of two 511 keV gamma photons in opposite directions. A PET scanner is equipped with a large number of scintillation detectors arranged in a ring surrounding the object of interest. When two opposing detectors register a photon simultaneously (coincidence detection), the line along which the annihilation took place (line of response) can be determined. A PET scan consists of the collection of large numbers of these coincidence events, which take place over time. Using mathematical reconstruction methods, the location of these coincidences, and thus the location or distribution of the compound, can be calculated as a function of time.74

At present this technique is used in various applications in physiology, biochemistry and pharmacokinetics. PET is a powerful diagnostic tool, particularly in oncology, neurological disorders and cardiovascular disease (for review, see Cherry74). It also has significant advantages. Firstly, it is possible to acquire kinetic data with a time resolution of a few seconds. This offers the possibility to image relatively rapid physiological and pharmacological processes. Furthermore, PET is exquisitely sensitive, having the ability to detect picomolar or even femtomolar concentrations of ligand in the tissue of interest. A third advantage is that it is quantitative, providing the potential to measure physiological or pharmacological parameters in absolute units. Finally, PET is relatively non-invasive, often requiring no more than vascular access for the injection of the radioligand or tracer.75

During the last few decades, the spatial resolution of PET scanners has steadily improved, which makes it now possible to scan even small animals such as rats and mice. This opens up a whole new area of research, although it also raises some practical issues. First of all, despite improvements in spatial resolution, there is a limit to the area that can be studied. Furthermore, during experiments, animals have to be fixed in the same position. Therefore, they are scanned under anaesthesia, which implies that the anaesthesia chosen should not interfere with the receptor of interest. In case of the GABA_A receptor only the combination of ketamine base with medetomidine hydrochloride can be used, as it was shown that, in contrast with other agents, this type of anaesthesia does not interfere with the GABAergic system.76–78

To quantify properties of the GABA_A receptor using PET, [11C]flumazenil is the most commonly used ligand. Flumazenil is a benzodiazepine antagonist, which is not selective for GABA_A receptor subtypes.79 Several methods have been developed
for quantifying receptor properties using PET. As these methods do not use the complete receptor occupancy range, only the ratio of $B_{\text{max}}$ over $K_D$ can be measured in a single experiment. When multiple scans in the same subject are performed, yielding different levels of receptor occupancy, estimates of both $B_{\text{max}}$ and $K_D$ can be obtained. It would be best to use the complete range of receptor occupancies to obtain most reliable estimates for both $B_{\text{max}}$ and $K_D$. For most ligands it is not possible to obtain this information, but, fortunately, as flumazenil is neither pharmacologically nor toxicologically active, this allows administration of saturating doses, yielding receptor occupancies up to 100%.

Thus, PET using $[^{11}\text{C}]$flumazenil can yield information on $B_{\text{max}}$ and $K_D$ of the GABA$_A$ receptor. When kinetics of flumazenil in blood are also measured, the distribution from blood to brain can be studied as well, providing insight into blood-brain barrier transport.

2.5.3 Electric cortical stimulation and behavioural analysis
An epileptic seizure involves a variety of phenomena, depending on seizure origin, the brain structure involved, and age. Seizures can present as a short loss of awareness (staring and disruption of ongoing normal activity), automatisms (undressing, chewing, repeated movements, etc), sensations (visual, sensory, auditory, olfactory, or gustatory), or motor phenomena. Motor events can either be uncontrolled rhythmic movements in face or limbs (myoclonic seizures), stiffening (tonic seizures), or sudden loss of muscle tone (atonic seizures).

Behavioural manifestations of spontaneous or evoked seizures in different animal models of epilepsy show remarkable similarities with clinical seizures in humans. Loss of awareness, or arrest, chewing, head nodding and clonic and tonic seizures are reported in a variety of animal models. Due to this similarity between clinical and experimental seizures, these behavioural phenomena are interesting tools in the study of epileptogenesis and pharmacoresistance. This is particularly interesting because the different types of seizures are known to originate in different brain regions (for review, see Véliková). The origin of clonic seizures is in the forebrain. These seizures also occur in models with seizure origin in limbic structures, such as the kainic acid model, but in the latter case the occurrence of these clonic seizures is a sign of activation of structures beyond the limbic system, namely the thalamus, neocortex, and basal ganglia. Anatomical substrates responsible for tonic-clonic seizures involve brainstem structures. In models in which the GABA$_A$ receptor system is involved and in limbic seizure models tonic-clonic seizures represent spread of paroxysmal activity from forebrain to brainstem.

In the present investigations, seizures evoked by electrical stimulation of the cortex were analysed using behavioural component analysis. Using a certain stimulation protocol, as described by Voskuyl et al, an epileptic convulsion is elicited. As this convulsion is characterised by a typical pattern of behavioural components, thresholds to elicit certain components such as forelimb clonus can be determined. This threshold
declines on repeated stimulation sessions to a stable level. Furthermore, administration of antiepileptic drugs increases the threshold in a concentration dependent manner. These results suggest that measurement of the threshold at which forelimb clonus can be elicited is a good predictor for quantifying the excitability of the brain, which might be decreased during epileptogenesis.

Another interesting feature of the cortical stimulation procedure is the possibility to study different motor components that are present during a seizure. Application of this procedure has the advantage that it is not necessary to wait for the development of spontaneous seizures and that a seizure is induced in a controllable manner. As stated above, the different behavioural manifestations of seizures originate from different brain structures. Browning and Nelson showed that typical facial and forelimb clonic activity was eliminated in pentylenetetrazol-induced seizures in rats after separating the forebrain from the brainstem. These animals were, however, still capable of displaying tonic flexion and extension. This strongly suggests that the origin of face and forelimb clonus is in the forebrain, whereas tonic seizures may originate from the brainstem. In pharmacological studies suppression of specific behavioural seizure components was studied on administration of different antiepileptic drugs. For instance, Jonker et al reported that lamotrigine did not affect eye closure although it suppressed various other ictal signs in a concentration-dependent manner, whereas midazolam suppressed forelimb clonus less potently than other ictal signs. This suggests involvement of different neuronal pathways in the antiepileptic action of different antiepileptic drugs.

In summary, electrical stimulation of the cortex might yield interesting biomarkers for epilepsy progression and pharmacoresistance. Several studies showed its usefulness in providing quantitative information on brain excitability. It can also be used to obtain information on seizure components in a controllable manner and even during the silent period between initial event (status epilepticus) and spontaneous seizure development. It is possible to repeat measurements, which is very attractive when studying disease progression and development of pharmacoresistance.

### 2.6 Modelling and predicting disease progression in epilepsy

In general, pharmacoresistance reflects a decrease in efficacy of antiepileptic drugs. Describing the time course of this decrease in efficacy quantitatively, preferably in relation to the rate of disease progression, this may lead to prediction of pharmacoresistance, and evaluation of the effect of an intervention. To be able to describe pharmacoresistance development quantitatively, firstly the pharmacokinetic-pharmacodynamic (PK-PD) relationship of the antiepileptic drugs should be determined. This will obtain a measure for the efficacy of the drugs. Repeating this at different time points during disease progression results in quantification of a possible decrease in effectiveness of a drug over time. Secondly, the time course of disease progression should be quantitatively determined using meaningful markers for the progression, preferably in association with changes in effects of the antiepileptic drugs. When subsequently the decrease in efficacy...
of antiepileptic drugs is related to the time course of the disease status, development of pharmacoresistance can be predicted and the optimal strategy for interventions can be designed.

PK-PD models have been developed for characterisation of the time course of drug effects. These models are based on the fact that the intensity of many pharmacological effects is a function of the amount of drug in the body and more specifically the concentration of drug at the effect site, the site of action. In conventional PK-PD analyses, model parameters that determine the status of a biological system in the absence of a drug are (kept) invariant with time, and physiology is generally considered constant at baseline. For progressive, chronic diseases, this is not a realistic description because biological functions may deteriorate over the time course of the treatment period. Therefore, more and more attention is being paid to development of mathematical models describing changes in the disease status over time. These models can be used to determine the influence of a drug on disease progression. Conversely, the effect of changes in biological functions, due to disease progression, on the efficacy of drugs can also be quantified using this method.

Another important factor to consider is the high degree of variability which often is observed between people with epilepsy. For several reasons, it is important to estimate this variability explicitly or to separate it from unexplained intra-individual variability. Firstly, the underlying general relationship can better be estimated, thus providing a more reliable model for predicting either drug effects, or development of pharmacoresistance and disease progression. Furthermore, when explicitly estimating this variability in terms of which parameters of the model vary within the population, the relation between these parameters and individual changes in biological functions can be investigated. This will result in a better insight in the causes of pharmacoresistance development and disease progression.

### 2.6.1 Models for description of pharmacokinetics, pharmacodynamics, and disease progression

The most widely used pharmacokinetic models are the compartmental models, in which a drug is assumed to be distributed into one or more interconnected hypothetical compartments, which mimic drug absorption, distribution, and elimination processes. Although these models are rather descriptive, the main advantage is that they provide a continuous description of the concentration. As most drugs have their target site in an organ or a peripheral tissue, rather than in plasma, distribution to the site of action may cause a delay in the onset of the effect. Therefore, distribution to the site of action must be accounted for in the model as well. In general, this is achieved by using the so-called effect compartment model.

Having obtained a continuous description of the concentration at the target site, this concentration subsequently is linked to the observed effect. A wide variety of pharmacodynamic models have been developed, differing in complexity, and
physiological and mechanistic background. The classic and most commonly used model is the sigmoid $E_{max}$-model, which is an empirical function for describing non-linear concentration-effect relationships. According to classical receptor theory, however, drug action results from two independent components: 1) an agonist-dependent component, which describes the interaction between drug and biological system in terms of target affinity and activation, and 2) a biological system dependent component, which is determined by receptor concentration and nature of the stimulus-response relationship. Based on receptor theory, the first component, describing the drug-stimulus relation, is a hyperbolic function, the shape of which is determined by the intrinsic efficacy of the drug and the affinity of the drug for the target. The intensity of the subsequently induced response is assumed to be a function of the stimulus. The relation between stimulus and response, the so-called transducer function, may in principle have any shape. For an extended review on the incorporation of the receptor theory in PK-PD modelling, the reader is referred to Danhof et al.\textsuperscript{95}

A unique property of this approach is the ability to describe simultaneously the effect of a variety of compounds acting on the same receptor system using a single PK-PD model. This has, for example, been to drugs activating the GABA\textsubscript{A} receptor.\textsuperscript{100} Visser et al developed a single PK-PD model which was able to describe the concentration-effect relationship of different GABAergic modulators, such as benzodiazepines and neurosteroids. Administration of benzodiazepines results in a concentration dependent increase in the amplitude of the $\beta$-frequency of the EEG to a maximum value at higher concentrations.\textsuperscript{70} The effects of neurosteroids, however, are more complex, as they initially increase the amplitude of the $\beta$-frequency of the EEG to values exceeding the maximum effect observed with benzodiazepines, but at maximal concentrations this amplitude decreases, even to below baseline values. This behaviour could be described by a single PK-PD model with a hyperbolic function, describing the receptor activation process, in combination with a biphasic transducer function, signifying the stimulus-response relationship.\textsuperscript{100,101}

Typically, drugs are administered to subjects suffering from a disease. If the disease is dynamic in nature, as is the case of many epilepsies, the biological system itself is changing over time, which might interfere with observed drug effects. On the other hand, (chronic) drug administration might change disease progression. Therefore, analysis of disease progression has been proposed.\textsuperscript{97,102} Disease progression can be analysed at different pathophysiological levels. Post et al distinguished three different levels.\textsuperscript{97} Firstly, at a molecular level, genetic, transcription and receptor mediated changes play a role. This results in an altered cell/tissue response, which is defined as the second level. Finally, changes in clinical endpoints comprise the third level. Based on available data, any one of these levels, or relationships between different levels can be analysed. Moreover, the relationship between disease progression and drug action can be identified. For instance, changes in the expression or functioning of the drug target over time may affect drug efficacy, thus leading to pharmacoresistance. On the other hand, the drug can affect the
disease status over time, either symptomatically, or protectively. By definition, protective drug effects can reduce, halt, or even reverse the disease process, whereas symptomatic treatments can only reduce symptom severity. \(^{97,102}\)

### 2.6.2 Population or mixed effects modelling

When quantifying drug effects and disease progression in patients, variability between subjects and even between different measurements within a single subject should be taken into account. One approach is population or mixed effects modelling as introduced by Sheiner and colleagues. \(^{103,104}\) A population model comprises two sub models: a structural model, describing the time course of drug concentration or effect, or disease progression, and a statistical model, which quantifies the variability within the study population. \(^{105}\) This variability is divided into two levels: the first level concerns variability between subjects, whereas the second level includes intra-subject or residual variability. Variability between subjects, or inter-individual variability, is expressed in terms of variability of structural model parameters. For instance, when a drug is less efficacious in some patients than in others, the maximal effect \((E_{\text{max}})\) of the drug might be lower in these patients, which will result in variability in \(E_{\text{max}}\) within the population. When the receptor density is also measured in the population, this information can be included in the model as a covariate. \(^{105}\) In that case, the relationship between the individual value of \(E_{\text{max}}\) and the measured number of receptors can be investigated.

As well as the possibility to include covariate effects in the model, population modelling has additional advantages. Firstly, simultaneous analysis of all data from a population increases the statistical power of estimating all model parameters. Secondly, incomplete datasets from individuals can be included, because the structural model is determined using the complete population.

Taken together, determining the PK-PD relationship of antiepileptic drugs during development of epilepsy, together with quantitatively describing the time course of epileptogenesis, is a useful tool to analyse and predict pharmacoresistance. In addition, when using population modelling, variability between subjects or measurements can be quantified and can, moreover, be related to covariates. This makes it possible to investigate what might underlie the observed variability within a population.

### References


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