Pharmacological and electrophysiological study of antiepileptic drugs in a chronic and acute model of epilepsy

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1999

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine, Institute of Neurology, University College London

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This work is dedicated to Mum and Dad.

"Destiny is not a matter of chance, it is a matter of choice; it is not a thing to be waited for, it is a thing to be achieved."

--William Cullan Bryant
ACKNOWLEDGEMENTS

I would first like to thank both my supervisors, Dr. Philip Patsalos, University Department of Clinical Neurology, Institute of Neurology, Queen Square, and Professor John Jefferys, Department of Physiology, University of Birmingham, for their help and advice over the last few years.

I also would like to express my sincere gratitude to Dr. Miles Whittington, Department of Physiology and Biophysics, St. Marys Hospital Medical School, and Dr. Neville Ratnaraj, University Department of Clinical Neurology, Institute of Neurology, London. Both for the unlimited generosity in their time, their invaluable help, assistance and advice and most of all for their encouragement during the times of misfortune and few results, and without whom this work would not have been possible.

In addition, I gratefully acknowledge the Brain Research Trust for the grant used to finance both myself and all aspects of this study.

Finally, but by no means least, I wish to thank a very special friend of mine, my dear husband Uinsinn Finn for his understanding and encouragement during the difficult times, his companionship during the good times, and finally for his invaluable support and love. "L’amour vient de l’aveuglement, l’amitie de la connaissance."--Comte de Bussy-Rabutin
ABSTRACT

The objectives of this theses were (i) to determine the kinetics of carbamazepine (CBZ), a well established antiepileptic drug and levetiracetam, a new antiepileptic drug presently undergoing clinical evaluation in a freely behaving rat model, (ii) to test the efficacy of these drugs on electrographic seizures in animals injected with tetanus toxin, and to determine the relationship between their efficacy and their corresponding concentrations in blood and cerebrospinal fluid (CSF), and finally (iii) to evaluate the antiepileptic properties of levetiracetam on bicuculline induced epileptiform bursts in the hippocampal slice.

The first objective entailed the development of a freely behaving rat model which allowed concurrent blood and CSF sampling and consequently pharmacokinetic characteristics of a drug over a relatively chronic period (7 days). Under anaesthesia a cisterna magna catheter, for CSF sampling, a jugular vein catheter, for blood sampling, and an intraperitoneal osmotic minipump set to deliver CBZ or levetiracetam were implanted. CSF and blood samples were collected on days 1, 2, 4, 6 and 7 post-surgery at timed intervals and analysed for CBZ and carbamazepine epoxide (CBZ-E; the primary pharmacological active metabolite of CBZ) or levetiracetam content by high performance liquid chromatography. The serum and CSF concentration versus time profiles of CBZ and CBZ-E, exhibited biphasic characteristics; the first phase involved rapid appearance of CBZ and CBZ-E in blood and CSF compartments followed by a gradual increase until maximum concentrations were achieved. During the second phase, CBZ exhibited a marked acceleration in its metabolism (autoinduction), as indicated by a dramatic reduction and a subsequent gradual decline in both CBZ and CBZ-E blood and CSF concentrations. However, in contrast to CBZ, chronic levetiracetam administration was not associated with autoinduction. Following its acute and chronic administration, levetiracetam appeared in blood and CSF compartments and thereafter concentrations rose linearly until maximum concentrations were achieved.

The second objective related to the efficacy of CBZ and levetiracetam in the in vivo
tetanus toxin model of epilepsy. Under anaesthesia, tetanus toxin was injected, and a bipolar electrode placed in the hippocampus. Initial EEG recordings began 1-2 days post-surgery and continued for 5-7 days. A minipump with CBZ/levetiracetam was then implanted intraperitoneally and continuous EEG and video recordings were undertaken for a further 7 days. The animals developed a chronic limbic epilepsy, characterized by the occurrence of spontaneous interictal spikes, polyspikes, non-generalised and generalised seizures. Both CBZ and levetiracetam exhibited efficacy in this model, involving a reduction in the maximum number of seizures occurring per day and a reduction in the total number of generalised seizures over the period analysed. However, a statistically significant result was only achieved following administration of the highest dose of levetiracetam (16 mg/kg/h; p=0.0004). Furthermore, at all doses studied a significant reduction in the duration of generalised seizures was observed following administration of CBZ (p<0.0001) and levetiracetam (p<0.0001).

The final objective related to the efficacy of levetiracetam in an in vitro hippocampal slice model of epileptiform activity. Hippocampal slices were made epileptic via bath application of bicuculline and raised extracellular potassium. This activity took the form of trains of population bursts with a distinctive biphasic pattern lasting several seconds. Levetiracetam (200 and 400 µmol/l) significantly (p<0.0001) reduced the overall duration of these seizure-like events without influencing the biphasic pattern.

In conclusion this thesis demonstrates that CBZ and levetiracetam possess different kinetic characteristics. Whilst CBZ exhibited complex and undesirable kinetics, levetiracetam were simple and predictable. Additionally, although CBZ and levetiracetam significantly reduced seizure generalisation in vivo, a statistically significant reduction was only achieved with the highest levetiracetam dose studied. Levetiracetam also appeared to attenuate the prolonged ictalform discharges in the disinhibited in vitro slice. Overall, these findings suggest that whereas levetiracetam does not effect epileptogenesis per se it does reduce seizure severity and, particularly, seizure generalisation in vivo in predominantly disinhibitory models.
PUBLICATIONS


DOHENY, H.C., WHITTINGTON, M.A., JEFFERYS J.G.R., PATSALOS, P.N., 1998. Levetiracetam is an anticonvulsant in both a chronic and an acute disinhibition model of epilepsy. Epilepsy Research [In Press].
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<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>ACSF</td>
<td>Artificial cerebrospinal fluid</td>
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<tr>
<td>AED</td>
<td>Antiepileptic drug</td>
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<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Bicuculline methiodide</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
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<tr>
<td>CBZ</td>
<td>Carbamazepine</td>
</tr>
<tr>
<td>CBZ-E</td>
<td>Carbamazepine-10,11-epoxide</td>
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<tr>
<td>cm</td>
<td>Centimetre</td>
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<tr>
<td>Cmax</td>
<td>Maximum drug concentration</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>CSF</td>
<td>Cerebrospinal fluid</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<td>Da</td>
<td>Dalton</td>
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<td>DMSO</td>
<td>Dimethylsulfoxide</td>
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<td>EEG</td>
<td>Electroencephalogram</td>
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<tr>
<td>GABA</td>
<td>γ-amino-butyric acid</td>
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<tr>
<td>GAD</td>
<td>Glutamic decarboxylase</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>hrs</td>
<td>Hours</td>
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<td>Hz</td>
<td>Hertz</td>
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<tr>
<td>i.d.</td>
<td>Internal diameter</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
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<tr>
<td>IPSP</td>
<td>Inhibitory postsynaptic potentials</td>
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<td>IS</td>
<td>Internal standard</td>
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<tr>
<td>iv</td>
<td>Intravenous</td>
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<tr>
<td>K⁺</td>
<td>Potassium</td>
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<tr>
<td>l</td>
<td>Litre</td>
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<tr>
<td>LD₅₀</td>
<td>Dose of drug causing death in 50% of animals treated</td>
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<tr>
<td>mg</td>
<td>Milligrams</td>
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<tr>
<td>mM</td>
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<td>MgCl₂</td>
<td>Magnesium chloride</td>
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<td>Abbreviation</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>Na&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Sodium</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Disodium hydrogen orthophosphate</td>
</tr>
<tr>
<td>o.d.</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>PA</td>
<td>Peak area</td>
</tr>
<tr>
<td>PDS</td>
<td>Paroxysmal depolarisation shift</td>
</tr>
<tr>
<td>PO&lt;sub&gt;4&lt;/sub&gt;³⁻</td>
<td>Phosphate</td>
</tr>
<tr>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Potassium dihydrogen orthophosphate</td>
</tr>
<tr>
<td>RSA</td>
<td>Rhythmic slow wave activity</td>
</tr>
<tr>
<td>t½</td>
<td>Elimination half-life</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Time to peak drug concentration</td>
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<tr>
<td>U</td>
<td>Units</td>
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<tr>
<td>µl</td>
<td>microlitres</td>
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<td>µmol</td>
<td>micromoles</td>
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<tr>
<td>V</td>
<td>Volts</td>
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<tr>
<td>Vd</td>
<td>Volume of distribution</td>
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Chapter 1 Introduction

1.1 Epilepsy

Epilepsy is not a disease but a chronic neurologic disorder. It is defined as ‘a continuing tendency to recurrent seizures of primary cerebral origin’. A seizure or epileptic attack is the consequence of a paroxysmal uncontrolled discharge of neurons within the central nervous system (CNS). Concomitant with this discharge, which can be recorded electrophysiologically, there are stereotyped paroxysmal alterations in behaviour. The clinical manifestations range from an almost instantaneous disturbance of sensation, loss of consciousness, impairment of psychic function, convulsive movements or some combination thereof.

Epilepsy is a term derived from a Greek word that means ‘to take hold of, to seize, or to possess’. This reflects the ancient Greeks’ belief that an epileptic attack represented possession by the gods. By about 400 BC the Hippocratic writers identified epilepsy as a physical disorder of the brain. John Hughlings Jackson, one of the founders of modern neurology, portrayed a seizure as a ‘sudden, excessive and temporary nervous discharge... of a few cells which have got far above the rest of the cortical cells in degree of tension and instability....’ (Jackson, 1931). The very earliest ideas now sound naive, possession by demons does not have much currency in mainstream epileptology today. More recently, epilepsy has been described as ‘a group of essentially electrical disturbances of brain function’ with ‘recurrent episodes of hypersynchronous neuronal activity in one or more of the cortical areas of the brain’ (Jefferys, 1993). Usually periods of more or less normal electroencephalographic (EEG) activity and behaviour are disrupted by episodes of gross electrical disturbance (Jefferys, 1993).

Next to vascular disorders, seizures are the commonest group of problems encountered in clinical neurology. Epilepsy affects about 2% of the population at some stage in their lives (Hopkins, 1987). The incidence of epilepsy is 20-50 per 100,000 per year. The prevalence of epilepsy is 4-10 per 1,000 people. The highest incidence of epilepsy occurs among the age groups of <1-4 years. Of all new cases
of epilepsy, 77% occur before the age of 20. Epilepsy can be hereditary or caused by damage or insult to the CNS. There are a huge variety of risk factors and events which can lead to epilepsy. Abnormal cellular discharge may be associated with a variety of causative factors including trauma, ischemia, tumours, infection and metabolic derangements. Clinically, the majority of cases are idiopathic but with newer forms of imaging (e.g. Magnetic Resonance Imaging, MRI), with genetic studies, and a huge quantity of experimental research, the underlying causes of these are becoming more apparent.

1.1.1 Classification of epilepsy

There are a number of classification schemes for epileptic seizures. The contemporary classification considers several aspects of the disorder. The first is the clinical expression of the seizure such as the events observed during the attack and the symptoms experienced by the patient. The second relates to the anatomic and physiological substrates of the seizure, based on the nature of the attack and on knowledge of brain function. Other considerations - aetiological, pathological, and age factors - may be relevant to the seizure state and may incorporate knowledge of genetic inheritance or acquired disease. Although a completely satisfactory seizure classification is not available, the Classification of Epileptic Seizures of the International League Against Epilepsy is the most widely accepted (Shorvon, 1990). It is based upon the nature of the attack rather than the presence or absence of an underlying cause. The use of the electroencephalogram (EEG) has greatly increased our understanding of the source or ‘point of origin’ of any particular type of epileptic attack.

![Epilepsy diagram]

Epilepsy

\[\text{Partial (60%)} \quad \text{Generalised (40%)}

\[\text{Simple} \quad \text{Complex}

Figure 1.1 Simplified classification of epilepsy
The simplest classification reflects the extent of the brain affected. From the above diagram, it can be seen that epilepsy is divided into two main categories - partial, (or focal) seizures and generalised seizures. Partial seizures, the commoner form, begin locally in one hemisphere and often remain locally (simple), but sometime spread to the other hemisphere (complex). When the spread of epileptic activity includes the other hemisphere, consciousness is lost. Partial seizures make up about 60% of all epilepsies. The clinical manifestations of partial seizures reflect the region of the brain involved. For example, an epileptogenic focus located in the motor cortex results in involuntary twitching initially of the fingers and face. Commonly, there is a sequential activation of different muscle groups as the abnormal electrical activity spreads from the focus to neighbouring cortical tissue. Thus, the motor activity may involve first the fingers, followed by wrist, elbow, shoulder, and eventually the face and leg. Generalised seizures or non focal epilepsy, however, begin with immediate involvement of both hemispheres. Seizures of either category, partial or generalised, differ substantially in their pharmacology, cellular physiology and their clinical manifestations (Jefferys, 1990).

Approximately 10 to 25% of seizures may remain unclassified, because they fail to fit into an existing category, or because descriptive details are lacking. Much work has been applied to the classification of seizures (Dreifuss, 1985). However, a definitive classification of seizures will probably not be possible until the pathophysiology of seizures is better understood.

1.1.2 The cellular physiology of epilepsy

In both animal models of epilepsy and in man, cortical neurons exhibit characteristic abnormalities of membrane potential and firing patterns. The first characteristically abnormal electrical event is the intermittent appearance of high voltage negative waves on the EEG (Figure 1.2). These are called interictal spikes because they resemble the spikes seen in the EEG between actual seizures in humans. As the interictal spikes become more frequent they become associated with a negative wave of slower time course. Collectively the fast (spike-like) and slow components are
referred to as the interictal EEG paroxysm. The slow negative component may also be associated with low voltage fast waves riding on the crest. When a full blown seizure occurs it typically arises from these fast components.

![Diagram of EEG events in seizure disorders](image)

Figure 1.2 Schematic presentation of neurological events in seizure disorders. (Adapted from Ayala et al., 1973)

The interictal EEG paroxysm provides a convenient and simple model for elucidating the electrophysiological mechanisms of epilepsy. Intracellular recording from neurons in an experimental epileptic focus show cellular discharges during the interictal spike that are driven by a large depolarisation called the paroxysmal depolarisation shift (PDS). The paroxysmal depolarisation shift is followed by a hyperpolarisation. There are two hypotheses regarding the cellular mechanisms of the paroxysmal
depolarisation shift. According to one, the paroxysmal depolarisation shift is a giant form of excitatory postsynaptic potential. Synchronous excitatory potentials summate to generate the observed depolarisation shifts. The excitatory feedback circuits, ubiquitous in the cortex, are thought to provide the explosive and synchronous generators for depolarisation shifts. Feedback inhibitory circuitry is thought to contribute to the hyperpolarisation observed after a paroxysmal depolarisation shift. It is easy to see how the normal balance between feedback excitation and inhibition could lead to epileptic activity when biased towards excitation. An alternative interpretation supported by more recent cellular studies by Prince (1986) and by Llinas (1986) is that the paroxysmal depolarisation shift results from active membrane processes intrinsic to a cell. According to this view, synaptic inputs serve only to trigger and synchronise this process across a population of cells. Dendritic spike generating mechanisms involving Ca\(^{2+}\) currents are thought to be important for this mechanism.

1.2 The rodent hippocampus as a tool in epilepsy research

An epileptic fit is a rather uniform reaction of brain tissues to a diverse variety of stimuli, including (among others) fever, infections, ionic and chemical disturbances, drug withdrawal, malformations, dysgenetic disorders and tumours. Nearly all areas of the human brain can be recruited in an epileptic discharge, cortical structures being predominantly involved. Studies on various species have demonstrated, epilepsy is not restricted to the human brain. This opens animal research to the modelling of epileptic disorders. In animal models the hippocampus has been shown to be especially susceptible to seizures, mirroring its importance in temporal lobe epilepsy.

The hippocampus has many important advantages in modelling epilepsy due to its cytoarchitecture. The cellular organization in three layers, characterises the hippocampus as part of the cortex, but phylogenetically an older part of the brain than the neocortex. The main structuring principles and the physiology of the hippocampus are conserved throughout several species, from man to many other mammals, including rodents. This allows the use of the rodent brain to mimic certain epileptic conditions. Although the relative size, location and neuronal connections to
surrounding areas differ between the rodent and human brain, conclusions can be
drawn with regard to possible clinical implications. In the following section the
anatomy and electrophysiology of the hippocampus will be explained with special
reference to the rat, the animal used in this study.

1.2.1 Anatomy of the hippocampus
The hippocampus, sometimes called Ammon’s horn, got its name from its
resemblance to a sea horse. It is probably among the best characterised cortical
structures and its highly regular organisation is ideally suited for anatomical and
physiological investigations. Much is known about the synaptic organisation and the
functional characteristics of its neurons. The hippocampus plays a key role in certain
aspects of learning and memory.

In the rat, as in rodents in general, the hippocampus is a cylindrical structure whose
longitudinal axis forms a semicircle around the thalamus. The hippocampus proper
can be divided into four regions, which have been traditionally called CA1-CA4
(Figure 1.3). The dentate gyrus, the subiculum and the entorhinal cortex are also
included in the term hippocampal formation. The CA1 and CA3 regions constitute
most of the hippocampus proper. The CA2 region is so small and indistinct in some
species that it is often ignored. Both the dentate gyrus and the CA3 region are three
layered cortices. The three fundamental layers of the hippocampus are the
polymorphic layer (the stratum), the pyramidal layer (the stratum pyramidale) and the
molecular layer (the stratum radiatum). The dentate gyrus consists of a polymorphic
layer (the hilus), a granular layer (the stratum granulosum) and a molecular layer
(stratum moleculare). The molecular layer of the dentate gyrus is continuous with that
of the hippocampus.

The principal and most numerous class of neurons in the hippocampus are the
pyramidal neurons. While the regular arrangement and shape of the pyramidal cells
determine the overall shape of the hippocampus they are not the only ones present.
In all layers there are cells of other types with short axons which take various courses.
Figure 1.3 Schematic diagram illustrating some main features in the organisation of the hippocampal formation. Labelled areas include the subiculum, part of the entorhinal cortex, dentate gyrus, and regions CA1 to CA4. Elements and layers in the dentate gyrus and the hippocampus are shown. Inset, illustrates a main path of transmission of impulses entering from the entorhinal area in fibres of the perforant path.
Among these are the basket cells, found in the stratum oriens. The basket and other cells are concerned with the intrinsic activity of the hippocampus. The efferent fibres leaving the hippocampus in the fornix are axons of the pyramidal cells. The main cellular principal neurons of the dentate gyrus are the granule cells. Afferent fibres to the hippocampus enter it from the entorhinal area (medial and lateral perforant path (Figure 1.3).

The main inputs to the hippocampus and dentate gyrus arise from the entorhinal cortex, the septal region and the contralateral hippocampus. The entorhinal cortex provides a major sensory input via the fibres of the alvear and perforant pathways. The entorhinal cortex itself receives input from many other regions of the brain such as fibres from the association cortices, the olfactory cortex, several thalamic nuclei, the claustrum and the amygdala. The second significant input from the septal region enters the hippocampus by four routes: the fimbria, the dorsal fornix, the supracallosal stria, and the amygdaloid complex. The terminations are distributed throughout the hippocampus, but preferentially in region CA3 and the dentate gyrus. A third sizable input consists of commissural fibres from the contralateral hippocampus. These fibres enter the fimbria and then collect in the fornix.

The hippocampus has several output pathways and targets. One major output proceeds via the fimbria, which is a sheet composed of the axons of pyramidal cells and cells in the subiculum (which in turn receive input from the hippocampus proper). These axons then gather to form the fornix, which crosses the midline of the brain. The main output of the dentate gyrus is to region CA3 via the mossy fibre axons.

1.3 Animal models of epilepsy

Most of what we know about the science of epilepsy has derived from animal models (Fisher, 1989). However, experimental models are not identical to epilepsy. They show mechanisms of models; not necessary mechanisms of epilepsy. Because the EEG and the behavioural picture of a model looks similar to a clinical seizure type,
this does not necessarily mean that the pathophysiology is the same. Although animal models have their drawbacks, they are, however, the best method available for the study of epilepsy at the cellular and neuronal network level. A large number of model systems exist. There are multiple types of the epilepsies to model. None of the models reflect in total clinical epilepsy. Important findings therefore require validation in several models.

Acute, chronic and genetic models of epilepsy may be selected for the study of epilepsy. The choice of a particular model depends on the nature of the study, the questions posed, and hence the kinds of samples, recordings and observations required. Acute models typically use convulsant drugs or changes in extracellular ion concentrations. They can only assess specific symptoms, e.g. interictal spikes or seizures, rather than epileptic states. Chronic models typically involve implanted heavy metal compounds, injected toxins, local lesions, or repeated stimulation. These treatments lead to a prolonged state of increased seizure susceptibility, or to recurrent spontaneous seizures. Chronic experimental models of epilepsy can be compared more closely with clinical epilepsy than can acute models. Furthermore, they are better suited for behavioural and EEG studies in freely moving animals. However, these models are more difficult to study at the level of cells and circuits (Jefferys, 1990). Genetic models depend on identifying epilepsy-prone states in the natural population (e.g. photogenic baboon) or artificial selection for epileptic traits in laboratory species (e.g. tottering mouse, genetically epilepsy-prone rat). Epilepsy occurs spontaneously in a variety of domestic and wild animals, for example dogs, some baboons and rodents. These animals have been used in epilepsy research (Delgado-Escueta et al., 1986).

Complex partial seizures are by far the most commonest type of seizure (Gloor et al., 1982). They usually arise from the limbic lobe, including amygdala, hippocampus, and less often, temporal neocortex or extratemporal structures (Gloor et al., 1977). Previously, complex partial seizures were referred to as psychomotor or temporal lobe seizures, as they almost always arise in the temporal lobe and they present most
of the physiological consequences of epilepsy. The four primary models of complex partial seizure are: kainic acid (Lothman et al., 1981; Lothman & Collins, 1981; Cavalheiro et al., 1982; Ashwood et al., 1986), tetanus toxin (Mellanby et al. 1977; 1981; 1984; 1985; 1993; Hawkins & Mellanby, 1987), kindling (Delgado & Sevillano, 1961; Goddard & Douglas, 1975; Rondouin et al., 1980) and the in vitro hippocampal slice (Schwartzkroin & Prince, 1976; 1977; 1978; 1980; Schwartzkroin, 1986). The work described in this thesis involved the use of two models namely the in vivo tetanus toxin model and the in vitro bicuculline model. Further details of other animal models go beyond the scope of this introduction and have been reviewed elsewhere (Fisher, 1989).

1.3.1 Chronic disinhibition: The tetanus toxin model

A model of recurrent, chronic partial seizures can be produced by injection of minute doses of tetanus toxin into rat or cat hippocampus (Mellanby et al., 1977; 1984). The epileptic syndrome in the rat resembles complex partial seizures in man in many respects (Mellanby et al., 1977). For instance, seizures occur spontaneously and intermittently, and generalised seizures are normally associated with clonic movements of the forelimbs which is commonly observed in human epilepsy. This model was categorized as a complex partial seizure model as a result of the location of the injection site in the limbic structures, rather than the properties of the toxin itself.

Tetanus is a disease (Weinstein, 1973) produced by a 145,000 Da toxin from a gram-positive bacteria, Clostridium tetani. In the disease state, toxin is transported from the periphery to the spinal cord, where it is believed to interfere with presynaptic release of inhibitory neurotransmitters (Price et al., 1975). In contrast, injection into hippocampus of a dose of toxin 3-6 times the mouse LD50 probably produces effects only locally (Mellanby et al., 1984). Seizures may occur within a day after injection and then on a chronically recurrent basis over weeks. A seizure in a rat typically begins with arrest of activity, followed by myoclonic jerks of the front limbs, and in some animals generalised tonic-clonic seizures (Mellanby et al., 1984). Whether or
not the seizure generalises depends upon several factors, including spread to the cingulate area (Hawkins et al., 1987). The experimental animal shows a wide range of behavioural abnormalities (Mellanby et al., 1977). Animals typically become increasingly irritated as the seizure syndrome progresses and resort to biting and leaping out of their cages at any attempt to handle them. The EEG shows concurrent 3-20 Hz spiking or spike-wave activity. These signs are similar to those of focal epilepsy in man. For approximately one month animals can have an average of about 8-20 seizures per day. Thereafter, the seizures decrease, such that the animals are seizure-free by several months after injection of tetanus. Therefore the epileptogenic activity is time-limited.

1.3.2 The mechanism of action of tetanus toxin

Tetanus toxin binds specifically to neuronal membranes, is transported axonally (Price et al., 1986) and acts as a protease on synaptobrevin which is part of the release apparatus for neurotransmitters (Mellanby & Green, 1981; Shiff et al., 1996). Its proteolytic effect on synaptobrevin, which is required for the docking and fusion of transmitter vesicles to the presynaptic membrane (Shiff et al., 1996), will affect equally excitatory and inhibitory cells. The higher firing rate of inhibitory neurons, resulting in a much faster cycle of transmitter vesicle release, is one likely reason for the inbalance of excitatory and epileptogenic action of tetanus toxin. Disturbances of the synaptobrevin mechanism will affect interneurons more rapidly and to a greater extent. To produce its effect tetanus toxin blocks preferentially the presynaptic release of GABA and glycine, two inhibitory neurotransmitters (Mellanby et al., 1977; 1981; 1984). Several studies reported a decreased release of the inhibitory neurotransmitter GABA and in the size of the IPSPS (Whittington & Jefferys, 1994; Jordan et al., 1991; Empson et al., 1993). These effects stem from the cleavage of synaptobrevin, a small vesicle protein, plays an important role for the function of synapses in being a key component of exocytosis (Li et al., 1996). Exocytosis is the process of fusion of secretory vesicles and plasma membranes and results in the discharge of vesicle content into the extracellular space and the incorporation of new proteins and lipids into the plasma membrane (Morgan, 1995). This process can be
constitutive or regulated (specialised cells such as neurons, endocrine and exocrine cells). Regulated exocytosis is usually, but not always, triggered by an increase in the cytosolic free Ca²⁺ concentration (Morgan, 1995). Two proteins of the the presynaptic plasma membrane, syntaxin and SNAP-25 and the synaptic vesicle membrane protein, synaptobrevin form a stable protein complex which is involved in the docking and fusion of synaptic vesicles in the mammalian brain presynaptic membrane (Shiff et al., 1996). Tetanus toxin has a remarkable specificity for synaptobrevin and cleaves three components in the synaptic vesicle protein complex, resulting in a decreased release in neurotransmitters. However, the epileptogenic effect of tetanus toxin outlasts the impairment of GABA release. Inhibition in the ipsilateral focus drops to 10% of controls during the first 2 weeks, but there is a subsequent recovery. The loss of inhibition in the contralateral mirror focus never drops below 50% (Empson et al., 1993; Whittington & Jefferys, 1994). These studies suggest a more complex action of the tetanus toxin and/or the induced focus than just the blockade of inhibitory transmission. One likely mechanism is that the inhibitory neurons are not recruited by the hippocampal network, either because they are less excitable or because the synapses that excite them are impaired (Bekenstein et al., 1993, Sloviter, 1991; Whittington & Jefferys, 1994).

The tetanus toxin model has a number of advantages over other experimentally-induced chronic epilepsy models. This model is well characterised (Mellanby et al., 1977; 1981; 1984; 1985; 1993; Hawkins & Mellanby, 1987) and has been proven very useful in screening antiepileptic drugs such as carbamazepine (Hawkins et al., 1985), phenytoin, valproic acid (Mellanby et al., 1985), piracetam (Hawkins & Mellanby, 1986) and oxiracetam (Brace et al., 1989). Injection of the toxin produces a chronic epileptic animal which suffers both generalised and non-generalised seizures randomly over several weeks. Although this model is a chronic model the epilepsy is somewhat reversible in that the animals eventually stop seizing and the abnormalities in their EEGs disappear (Mellanby et al., 1981). This reversibility makes it possible to distinguish between behavioural effects of ongoing epilepsy and long term consequences of the previous epilepsy. Finally, the effects of the toxin
<table>
<thead>
<tr>
<th>Convulsant agent</th>
<th>Mechanism of action</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bicuculline, picrotoxin, penicillin</td>
<td>Decreased synaptic inhibition</td>
<td></td>
</tr>
<tr>
<td>4-Aminopyridine (4-AP)</td>
<td>Increased synaptic transmission</td>
<td>Prolongs presynaptic action potentials</td>
</tr>
<tr>
<td>Low [Mg(^{2+})](_0)</td>
<td>Increased synaptic excitation</td>
<td>Unblocks N-methyl-D-asparate receptors</td>
</tr>
<tr>
<td>Subacute cholera toxin; cyclic AMP analogues</td>
<td>Increased synaptic excitation</td>
<td></td>
</tr>
<tr>
<td>Elevated [K(^+)](_0)</td>
<td>Increased neuronal excitability</td>
<td>Partial block of inhibition</td>
</tr>
<tr>
<td>Low [Ca(^{2+})](_0)</td>
<td>Non synaptic</td>
<td>Also via gap junctions in 4-AP with picrotoxin, CNQX, and APV</td>
</tr>
</tbody>
</table>

Table 1.1 Acute convulsant models and their corresponding mechanism of actions.
occur without any associated gross pathological changes at the site of injection (Mellanby et al., 1977).

Since the focal seizures occur at a consistent rate for 4-6 weeks (Jefferys et al., 1987), it is possible to monitor seizure frequency for several days before, during and after the start of the application of an anticonvulsant drug. Tetanus toxin therefore constitutes an excellent experimental model of epilepsy, and allows possible screening of novel treatments for complex partial seizures.

1.3.3 Acute disinhibition: Hippocampal slice preparation

Many models of epileptiform activity have been developed using in vitro slices, particularly the in vitro hippocampal slice preparation. Using this preparation, investigators have elucidated some of the intrinsic neuronal and synaptic properties that appear to be involved in the generation of burst activity and hyperexcitability typical of epileptic brain. Focal seizures can be induced experimentally in vitro by agents, with rather diverse means of action (Piredda et al., 1985; Stringer & Sowell, 1994; Traub & Jefferys, 1994). Typically agents used act by causing disinhibition. In general, the balance of excitation and inhibition has to be shifted towards increased excitation. Resultant epileptic bursts can be generated by modification of (i) intrinsic (Rutecki et al., 1987; Perreault & Avoli, 1992; Watts & Jefferys, 1993; Traub & Jefferys, 1994; Traub et al., 1995; 1996), (ii) synaptic (Dreier & Heinemann, 1991; Traub & Jefferys, 1994; Traub et al., 1996) and (iii) non synaptic (Jefferys & Haas, 1982; Taylor & Dudek, 1982) mechanisms (Table 1.1).

Blocking synaptic inhibition is a common mechanism for the induction of focal seizures. Such disinhibitory models include the application of convulsant drugs such as bicuculline (Heyer et al., 1981; Wong et al., 1986; Federico & MacVicar 1996; Ameri et al., 1997), picrotoxin (Traub et al., 1984; Knowles et al., 1987; Gean et al., 1993; Traub et al., 1993; Brodie, 1995), penicillin (Schwartzkroin & Prince, 1977; Swann & Brady, 1984; Schneiderman, 1986; Knowles et al., 1987; Stankiewicz et al., 1995; Traub et al., 1993) and pentylenetetrazol (Piredda et al., 1985; Bingman
Pharmacological blockade of GABA\textsubscript{A} -mediated inhibition in slices from adult rodents with these agents have been shown to induce brief synchronised population bursts followed by a series of shorter secondary bursts (after discharges), lasting a few hundred milliseconds overall.

1.3.4 **Mechanism of action of bicuculline methiodide**

As mentioned earlier, cortical structures such as the hippocampus and the cerebral cortex are considered to be particularly susceptible to seizure and epileptiform electrical activity, and as such are subject of intense investigation relative to hyperexcitability. The chemical agent, bicuculline, acts as a GABA\textsubscript{A} receptor antagonists. It antagonises GABA-mediated inhibition by binding to (Mohler & Okada, 1977) and displacing GABA from its neuronal receptor binding sites (Olsen et al., 1978). Bicuculline selectively blocks GABA-mediated inhibition in vivo in both vertebrates (Johnston et al., 1972; Curtis et al., 1974) and invertebrates (Ayala et al., 1973). Bicuculline binds to brain membranes (Mohler & Okada, 1977) and displaces \textsuperscript{3}H-GABA from high affinity binding sites on brain and spinal cord neurons in vivo (Olsen et al., 1978) and in cell culture (Heyer et al., 1981). Thus, bicuculline antagonises GABA-mediated inhibition by competing with GABA for binding to high affinity binding sites of GABA receptors.

Bicuculline when added to the slice bathing medium induces bursting activity and after discharges, similar to that induced by penicillin or picrotoxin (Schwartzkroin, 1986). This activity may be spontaneous, or triggered by electrical stimulation in the appropriate afferent pathways. This epileptiform activity also seems to be analogous to the inter-ictal spikes seen in *in vivo* experiments on hippocampus. These antagonists produce subtly different discharge patterns, probably because of differences in their means and efficacies in blocking synaptic inhibition. Bicuculline produces bursts of action potentials, somewhat longer than those produced by penicillin, and these bursts are also followed by long after-hyperpolarisations, probably due to a calcium dependent potassium conductance (Alger & Nicoll, 1980;
Schwartzkroin, 1986). A variety of authors have termed the burst discharge produced by these agents a “paroxysmal depolarisation shift (PDS), taking that term from the \textit{in vivo} literature dealing with epileptiform activity produced acutely by topical application of epileptogenic agents (Dichter & Spencer, 1969; Schwartzkroin, 1986).

1.4 Pharmacology

The word pharmacology comes from the Greek \textit{pharmakon}, equivalent to “drug”, “medicine”, and \textit{logia}, meaning “study”. It is the study of the properties of chemicals and living organisms and all aspects of their interactions. Pharmacology is a branch of biology, since it is concerned with living organisms, but is equally related to chemistry, seen that it deals with chemical agents. It is also an essential part of medicine. For although a drug, in broad terms, is any chemical agent other than food that affects living organisms, in its medicinal sense, a drug is any chemical agent used in the treatment, cure, prevention or diagnosis of disease. Pharmacology makes use of mathematics to express its principles in quantitative terms. Pharmacokinetics, on the other hand, is the science and study of factors which determine the amount of drug at sites of biological effect at various times after application of an agent to a biological system.

1.4.1 Pharmacokinetics

A fundamental hypothesis of pharmacokinetics is that a relationship exists between the pharmacological or toxic response to a drug and the concentration of the drug in a readily accessible site in the body. In most cases the concentration of drug in the systemic circulation will be related to the concentration of drug at its site of action. The pharmacological effect that results may be the clinical effects desired, a toxic effect, or in some cases, an effect unrelated to efficacy or toxicity. Pharmacokinetics attempts to provide both a more quantitative relationship between dose and effect and the framework with which to interpret measurements of concentrations of drugs in biological fluids. Important concepts of pharmacokinetics are derived from mathematical analysis of absorption, distribution and elimination of drugs. The concepts of biological half life, zero order and first order kinetics, volume of
distribution and clearance must be clearly understood for proper application to the clinical situation (Evans et al., 1986).

To produce its characteristic effects, a drug must be present in appropriate concentrations at its site of action. An understanding of pharmacological and pharmacokinetic principles as they apply to antiepileptic drugs is necessary in order to prescribe drug therapy for the maximum benefit of the patient with epilepsy. Important concepts of pharmacokinetics include drug bioavailability, absorption, distribution, metabolism, and excretion (Benet et al., 1984). By applying pharmacokinetic principles, one can make allowances for inter-individual differences in absorption, distribution, protein binding and elimination. Thus individualisation of potent therapy can be achieved (Mitchell et al., 1984).

The technological advances in antiepileptic drug monitoring of the past 20 years have enhanced our understanding of seizure disorders, mechanisms of action of antiepileptic drugs, and the pharmacology and pharmacokinetics of these compounds. The availability of accurate and reliable quantitative analysis of antiepileptic drugs in biological fluids and tissues has also led to significant improvements in the treatment of epilepsy. Extensive reviews of drug kinetics are available (Brodie, 1964; Mitchell et al., 1984; Benet et al., 1984; Evans et al., 1986; Wilder & Bruni, 1981). The following sections are summaries of the most relevant information taken from the above reviews.

1.4.2 Drug absorption
The movement of drugs across cell membranes can be achieved by various processes including passive diffusion, active transport, pinocytosis and filtration. However, these transport processes do not explain movement over greater distance. In fact the forces which drive passive or facilitated diffusion, active transport and pinocytosis, are sufficient only to move solutes across the very short span of cellular membranes. The movement of drugs over greater distance is achieved via the circulatory and lymph system. The procedure involved in getting a drug to its site of action involves...
two separate processes, absorption and distribution. Absorption is the movement of the solute into the bloodstream from the site of administration and distribution is the movement of solute from the blood into the tissue.

The rate at which a drug reaches its site of action depends on both its rate of absorption and its rate of distribution. These rates, are in turn, determined by the rate of translocation across the specific barriers interposed between the sites of absorption and action. The movement of drugs across biological membranes is largely determined by the physicochemical properties of the drugs and cell membranes across which the drugs are transported in their passage through the body. Factors which determine the ability of a drug to cross biological membranes include molecular weight, its lipid and water solubility, degree of ionization and pH, protein binding, and the presence or absence of active transport mechanisms. Absorption is also influenced by the available surface area and the flux of molecules across the biological membranes. The latter is affected by concentration and therefore by rate of digestion and also by rate of removal of absorbed substances in blood or lymph.

1.4.3 Drug distribution

Once the solute has reached the bloodstream, the principal pathway for its distribution, it usually must traverse one or more biologic barriers in order to reach its ultimate site of action. Its accessibility to the site is determined initially by its ability to transverse the capillary wall, then by the blood flow through the site and finally if the drug acts intracellularly, by its rate of passage across cells. Thus the sequence of movement of a solute in the process of its distribution is the reverse of that involved in its absorption. Patterns of drug distribution reflect certain physiological factors and physicochemical properties of drugs. The kinetics of drug distribution may influence the time of onset, peak effect, magnitude and duration of drug action. Drug distribution depends on regional blood flow, cardiac output, pH gradients, extent of plasma and tissue protein binding, and permeability of cell membranes (Shand et al., 1975). It influences the amount of available drug in the CNS and the rate of drug elimination.
The distribution of drugs to the CNS, from the bloodstream is unique, mainly in that entry of drugs into the cerebrospinal fluid and extracellular space of the CNS is restricted. However, this decreased permeability pertains only to the diffusion of water-soluble or ionized molecules. Cerebral blood flow is the only limitation to permeation of the CNS by highly lipid-soluble drugs. Lipid-soluble substances diffuse across brain capillaries at rates determined by their lipid/water partition coefficients, just as at other capillary barriers. In fact, since the brain receives one sixth of the total amount of blood leaving the heart, lipid soluble drugs are distributed to brain tissue very rapidly compared with tissue such as muscle. Water soluble materials for which active transport processes exist, such as glucose and amino acids, also gain rapid access to brain cells.

There are two routes by which anticonvulsants and most other drugs enter CSF and brain. Firstly, across the cerebral capillary endothelial cells, site of the blood brain barrier, into the brain interstitial space, thence either into CSF or into the neurons and glia. Secondly, across the choroid-plexus cells, site of the blood-CSF barrier, directly into CSF. The proportion of drug that goes by each route depends on a number of factors. These include the rate of blood flow to the cerebral capillaries, the total surface area per unit weight of brain, and the permeability of the endothelium of the capillaries as compared with the rate of flow to the choroid plexus and the surface area and permeability of its capillary system.

The body compartments in which a drug accumulates are potential reservoirs for the drug. If stored a drug is in equilibrium with that of plasma and is released as the plasma concentrations declines, a concentration of the drug in the plasma and its locus of action is sustained, and pharmacological effects of the drug are prolonged. However, if the reservoir for the drug has a large capacity and fills rapidly, it so alters the distribution of the drug that larger quantities of the drug are required initially to provide a therapeutically effective concentration in the target organ.
1.4.4 Drug metabolism

The process of absorption and distribution determine not only how but also how quickly a drug will reach its site of action; they determine the speed of onset of drug effect. If nothing else happened to a drug after it entered the body, its action would continue indefinitely. Although this would be quite advantageous in the case of epilepsy, it would not be so in most other illnesses. The interactions between a drug and the body are not confined to the changes which the drug brings about in the living organism; the body also acts on the drug. This interaction is variously referred to as drug metabolism or biotransformation. The chemical alterations of drugs are not spontaneous reactions but rather catalysed reactions. They take place only in the presence of enzymes, the protein catalysts which accelerate the action but remain apparently unchanged in the process. The liver is the organ principally responsible for drug metabolism.

The reactions that drugs undergo during biotransformation yield products which are almost invariably less lipid soluble than their parent compounds. By far the most important enzymes of drug metabolism are those of the microsomal fraction of cells. Most of the antiepileptic drugs e.g. phenytoin, phenobarbital, primindone, ethosuximide, carbamazepine and valproic acid, are metabolized by the cytochrome P450 system in hepatic microsomes. The chemical reactions of enzymatic biotransformation are classified as either phase-1 or phase-2 reactions. Phase-1 reaction usually changes drugs into more polar and less biologically active compounds by oxidation, reduction or hydrolysis (e.g. phenytoin, phenobarbital and ethosuximide). Some metabolites, however, have significant anticonvulsive properties (e.g. the metabolites of carbamazepine, primidone and valproic acid). Phase-2 reactions, which are also called conjugation or synthetic reactions, involve coupling the drug or frequently its polar metabolite with an endogenous substrate, such as glucuronate, sulfate, acetate, or an amino acid.

Theoretically, altered drug metabolism is predictable when the effects are a function of drug concentration and no active metabolites are formed. Increased drug
elimination, reduced steady state drug concentration and decreased duration of drug action all result from enhanced drug metabolism. Drug half life and total drug action will be decreased. The liver is the most important organ for antiepileptic drug metabolism: the lungs, kidneys and gastrointestinal tract also contribute to a minor degree.

1.4.5 Drug excretion
Excretion is the process whereby materials are removed from the body to the external environment. It is the process by which the body terminates the action of a drug. The principle route for drug excretion is by way of the kidney, e.g. 50-60% of phenobarbital may be eliminated by the kidneys, although drugs may be excreted in any media from the body. The renal mechanisms which account for the rate and extent of urinary excretion of drugs are identical with those which normally account for the formation and final composition of voided urine. Urinary excretion of drugs begins with glomerular filtration of any drug that is not bound to plasma proteins. In most cases, the final concentration of the drug in the voided urine is determined by how much is passively reabsorbed in its passage through the renal tubule. The concentration gradient for passive back diffusion is created by the active reabsorption of Na⁺ and the concomitant removal of water. The extent of reabsorption of the drugs is dependent on its lipid solubility and degree of ionization at the pH of the tubular urine. Active tubular secretory mechanisms account for the rapid elimination in the urine of certain organic acidic or basic drugs. Drugs and metabolites may also be partly excreted in bile by passive diffusion or active transport.

1.4.6 Clearance
Clearance is the most important concept to be considered when a rational regimen for long term drug administration is to be designed. The desired effect is to maintain steady state concentrations of a drug within a known therapeutic range. Steady state refers to a stable plasma drug concentration at a constant dose rate. Assuming complete bioavailability, the steady state will be achieved when the rate of drug elimination equals the rate of drug administration. Thus, if the desired steady-state
concentration of drug in plasma or blood is known, the rate of clearance of the drug will dictate the rate at which the drug should be administered.

It is important to note that clearance is not how much drug is being removed, but rather the volume of biological fluid such as blood or plasma that would have to be completely freed of drug to account for the elimination. Clearance is expressed as a volume per unit of time. Clearance of most drugs is constant over the range of concentration in plasma or blood that is encountered in clinical settings. This means that elimination is not saturated and the rate of elimination of drug is proportional to its concentration.

1.4.7 Biological half-life

The biological half-life of a drug is the time required for the drug concentration to decrease by 50% after absorption and distribution are complete. For most drugs, half-life is independent of route administration, plasma concentration and dosage. Knowledge of the biological half-life of a drug is important in determining the optimum dosage interval and the duration of pharmacological effect. For drugs metabolized by enzyme systems that can become saturated, it is important that half-life determinations be made at concentrations that do not saturate metabolizing enzymes; otherwise, the biological half-life increases. Half-life is generally determined by administrating a drug intravenously and measuring the time of its disappearance from plasma.

Knowledge of the half-life of antiepileptic drugs allows determination of an optimum dosage interval and the time required for a drug to reach steady-state plasma concentrations. Drugs with long half-lives and that lack significant gastrointestinal effects from single large oral doses, such as phenytoin and phenobarbitone, can be administered to adults in single daily doses. However, drugs with shorter half-lives, such as valproic acid, need to be administered in two or three divided doses to maintain therapeutic plasma concentrations over a 24 hr period. Mathematical analysis predicts that a minimum of five drug half lives must pass before a steady-
state plasma concentration is achieved. Thus, the effectiveness of a drug at a given
dose cannot be accurately evaluated until this time, and dosage adjustment must be
delayed.

1.4.8 First order kinetics
Most antiepileptic drugs are metabolized by first order kinetics. In this type of
reaction, the rate of metabolism varies directly with drug concentration. As drug
concentration increases, metabolism increases proportionately, and the half-life
remains constant. The plasma concentration of the drug also increases proportionately
with increasing dose.

1.4.9 Zero order kinetics:
In a zero order kinetic reaction, the rate of metabolism reaches a maximum until
enzyme saturation occurs; it then proceeds at a constant rate. The plasma drug
concentration increases disproportionately with increasing dose and the biological
half-life increases with increasing dose. Zero order kinetics are best demonstrated by
phenytoin. With daily doses of phenytoin above 4-7 mg/kg, a small increase in the
dose may result in a large increase in the plasma phenytoin concentration. The dose
at which hepatic enzymes become saturated, however, varies from patient to patient.
With the knowledge that phenytoin may exhibit zero order kinetics at high plasma
concentrations, the physician should make only small dosage adjustments. Too great
an increase in daily dose may lead to toxic plasma concentrations.

1.4.10 Volume of distribution
The volume of distribution (Vd), expressed in litres per kilogram, describes the
relationship between the amount of drug in the body and the plasma drug
concentration. This volume does not necessary refer to an identifiable physiological
volume, but merely to the fluid volume that would be required to contain all of the
drug in the body at the same concentration as in the blood or plasma. If a drug is
confined to the blood stream, its Vd is equal to the volume of blood. If a drug is
highly bound to body tissue, the apparent Vd may be much greater than total body
water. Thus, the degree of plasma protein binding can influence the apparent Vd. It is obvious that the plasma concentration of a drug is affected by dilution within the central compartment (in which the drug distributes rapidly), slower entry into the peripheral compartment, and rate of elimination from the body.

1.5 Antiepileptic drugs

As previously discussed, animal models are used to study many aspects of the pathophysiology of epilepsy. One important aspect is the study of the effect of antiepileptic drug treatment. An antiepileptic drug is a drug that, when administered over a prolonged period, will decrease the incidence or severity of spontaneous seizures occurring in patients with epilepsy. There are many antiepileptic drugs available to treat epilepsy. Different drugs are used in different types of epilepsy. The main first line drugs used are phenytoin, carbamazepine, ethosuximide, and sodium valproate. Second line drugs are phenobarbitone, primidone and various benzodiazepines. Examples of new drugs are vigabatrin, lamotrigine, topiramate and gabapentin.

There are two general ways in which drugs might abolish or attenuate seizures: through effects on pathologically altered neurons of seizure foci to prevent or reduce their excessive discharge, and through effects that would reduce the spread of excitation from seizure foci and prevent detonation and disruption of function of normal aggregates of neurons. The majority of the existing antiepileptic drugs act at least in part by the second mechanism, since all modify the ability of the brain to respond to various seizure evoking stimuli. Although a variety of physiological effects of such drugs have been noted, especially effects on inhibitory systems that involve GABA, it is increasingly difficult to define the exact effects that might be prominent at therapeutic concentrations of free drug in plasma. In fact the exact mechanisms of antiepileptic agents is poorly understood.

The ideal antiepileptic drug would obviously suppress all seizures without causing toxic effects. Unfortunately, the drugs available today not only fail to control seizure
activity in some patients, but they frequently cause side effects that range in severity from minimal impairment of the CNS to death from aplastic anaemia or hepatic failure. The two drugs investigated during this thesis were carbamazepine and levetiracetam.

5.1 Carbamazepine

Carbamazepine (CBZ) was approved in the United Kingdom for use as an antiepileptic agent in 1963. Like many antiepileptic drugs the development of CBZ came about indirectly. C.J. Geigy, Ltd. sought to develop a psychoactive drug following the introduction of the neuroleptic chlorpromazine by Rhone Poulenc. Researchers at Geigy, developed imipramine, which unlike chlorpromazepine, demonstrated antidepressant properties when tested in psychiatric patients. In further efforts to find a neuroleptic, studies were made of other tricyclics, carbamoyl compounds of an iminostilbene that had been synthesised earlier in the Geigy labs. One of these compounds CBZ, revealed even more potent anticonvulsant activity in various animal models of epilepsy. CBZ soon found its place as the treatment choice of all types of epilepsy. It is now considered to be a primary drug for the treatment of partial and tonic-clonic seizures.

1.5.2 Chemistry

CBZ is an iminostilbene derivative and a structural congenor of the tricyclic antidepressant drug imipramine (Figure 1.4), with a carbamyl group at the 5 position; this moiety is essential for potent antiepileptic activity.

![Chemical structure of carbamazepine](image)

Figure 1.4 Chemical structure of carbamazepine
It has an empirical formula of $\text{C}_{15}\text{H}_{12}\text{N}_2\text{O}$ and a molecular weight of 236.26 Da. It is a white crystalline compound with a melting point between 190 °C and 193 °C. It behaves as a neutral lipophilic substance; it dissolves in ethanol, chloroform, dichloromethane, and other solvents but is virtually insoluble in water. Aqueous solution can be made with propylene glycol in which the solubility is enhanced by moderate heating.

1.5.3 Anticonvulsant activity

CBZ is an important drug in the treatment of epilepsy, and its therapeutic usefulness is well documented (Rodin et al., 1974; Troupin et al., 1977; Kosteljanetz et al., 1979). CBZ has been shown to be effective in the management of generalised convulsive and focal (partial and complex partial) seizures (Troupin et al., 1977). However, it is ineffective against generalised absence seizures. CBZ is the drug of choice in the management of generalised and partial seizures in children with epilepsy (Gamstorp, 1975). CBZ is also effective in the single dose and the long-term treatment of manic-depressive illness (Post et al., 1984) and is the drug of choice for treatment of trigeminal neuralgia (Blom & Goulen, 1977).

1.5.4 Adverse effects

CBZ is generally a well tolerated antiepileptic drug. However, unwanted side effects have been reported. These include sedation, weight gain, nystagmus, gastrointestinal symptoms, change in affect and mood, tremor, cognitive disturbance, rash diplopia and impotence. The problems however, are similar and in some cases less to those encountered with other standard antiepileptic drugs. For example although CBZ is as potent as phenytoin it has fewer cosmetic side effects. Compared to valproate, tremor and weight gain are much less common.

The systemic adverse effects that most commonly occurs during initiation of therapy is nausea or gastrointestinal discomfort, which abates in most patients as pharmacodynamic tolerance develops and autoinduction lowers blood and brain concentrations. Neurotoxic adverse effects are primarily dose related and reversible.
During initiation of therapy, dizziness, diplopia, or blurred vision, sedation and headache are most common (Cereghino et al., 1974; Dodson et al., 1987; Herranz et al., 1988; Holmes et al., 1993; Mattson et al., 1985; Mattson et al., 1992; Rodin et al., 1974; Smith et al., 1987). Slow and gradual increase in dosage at start-up minimizes these effects. With long term administration these symptoms usually subside, unless maximum dosage is given due to difficulty in obtaining seizure control. Adverse neuropsychologic effects of CBZ are generally minimum.

1.5.5 Carbamazepine-10,11-epoxide
Carbamazepine-10,11-epoxide (CBZ-E), the primary metabolite of CBZ, is pharmacologically active and chemically stable. Purified CBZ-E is a white crystalline compound with the empirical formula C\textsubscript{15}H\textsubscript{12}N\textsubscript{2}O\textsubscript{2} (Figure 1.5) a molecular weight of 252.27 Da and a melting point with decomposition at 200 to 205°C (Bellucci et al., 1987). CBZ-E is a neutral, lipophilic compound that, like the parent drug, readily dissolves in organic solvents and has a very limited solubility in aqueous solution.

![Figure 1.5 Chemical structure of carbamazepine, 10, 11 epoxide](image)

CBZ-E has been shown, in various animal models, to possess similar anticonvulsant properties to CBZ (Faigle et al., 1975 (a); Frigerio et al., 1975 (b); Bourgeois et al., 1984) and equipotent to the parent compound in animal testing (Faigle et al., 1976). It is effective in the treatment of trigeminal neuralgia in patients (Bertilsson et al., 1986) and is stable and identifiable in blood, brain, and CSF following long-term dosing with CBZ (Friis, et al., 1978; McKauge et al., 1981). CBZ-E may contribute
to the neurological side effects of CBZ (Gillham et al., 1988; Patsalos et al., 1985; Warner et al., 1992). Laboratory evidence using the mouse as an experimental model suggests that anticonvulsant efficacy and toxic side-effects of CBZ correlate better with the sum of the concentration of CBZ and CBZ-E in the brain than with the concentration of either alone (Hoppener et al., 1980).

1.5.6 Levetiracetam
Levetiracetam is a nootropic agent currently undergoing late phase clinical trials, (Phase 11 and Phase 111), in Europe and the United States, as adjunctive therapy in patients with partial epilepsy. It was originally developed for the treatment of Alzheimer’s disease, but has now been discontinued for this indication. It is too early to ascertain the exact role of levetiracetam in the management of epilepsy, but preliminary results are encouraging and show that levetiracetam has significant efficacy in patients with complex partial seizures and that it is also well tolerated (Sharief et al., 1996). It is likely that levetiracetam will prove an important new anticonvulsant for the treatment of epilepsy.

1.5.7 Chemistry
Levetiracetam [ucb LO59, (S)-alpha-ethyl-2-oxo-pyrrolidine acetamide] is the S enantiomer of the ethyl analogue of piracetam, a nootropic agent widely used clinically as a cognition-enhancing agent in the elderly since 1972. It is a white crystalline powder readily soluble in water, with the empirical formula of C₈H₁₄N₂O₂ (Figure 1.6) and a molecular weight of 170.21 Da.

Figure 1.6 Chemical structure of levetiracetam
1.5.8 Anticonvulsant activity

Levetiracetam is a centrally acting drug that not only has potential therapeutic application in epilepsy (Gower et al., 1992; Loscher et al., 1993), but also in anxiety states, and or disorders involving cognitive disturbances (Neyens et al., 1995). Its mechanism of action is unknown although a recently reported novel specific binding site for [3H] levetiracetam, unique to brain, may be involved (Noyer et al., 1995). Previous studies with levetiracetam have explored the possibility of whether levetiracetam exerts its anticonvulsant effects via an interaction with GABA-ergic mechanisms (Margineanu & Wulfert, 1997; Loscher et al., 1996). It was concluded from these investigations that although some interaction could exist between the levetiracetam binding site and the GABA_{A} receptor complex, these interactions are possibly indirect. Such conclusions were reached because levetiracetam was found to be inactive at GABA_{A} binding sites in radioligand studies (Loscher et al., 1996), and in pharmacological studies levetiracetam did not interact directly with the GABA_{A}/benzodiazepine receptor chloride ionophore complex (Margineanu & Wulfert, 1997).

Our data somewhat confirm these results as levetiracetam's anticonvulsant effect in the tetanus toxin model is unlikely to be a direct effect on GABA_{A} as this would have expected to decrease the incidence of seizures much more.

Additionally recent studies carried out by Loscher et al., 1996, examined the effects of a wide range of anticonvulsant doses of levetiracetam on GABA levels and activities of its synthesizing and degrading enzymes in several rat brain regions. The GABA turnover in brain regions was determined at different time points after administration of this drug (Loscher et al., 1996). These data demonstrated that treatment with levetiracetam induced alterations in the GABA system in several brain regions. In addition since both increases and decreases in the GABA-synthesizing enzyme glutamic decarboxylase and the GABA-degrading enzyme GABA transaminase were found it is not likely that these alterations were direct effects of levetiracetam but rather a consequence of post-synaptic changes in either GABAergic or other neurotransmitter-related systems (Loscher et al., 1996).
The finding that levetiracetams antiepileptic activity was highly stereoselective led to the investigation of a possible presence of a specific binding site for levetiracetam in the rat CNS (Noyer et al., 1995). The results obtained from this study suggest that levetiracetam labels a single class of binding sites with modest affinity and with a high capacity, in brain regions such as the hippocampus, cortex, cerebellum and striatum (Noyer et al., 1995). The binding of levetiracetam is also confined to the synaptic plasma membranes in the CNS since no specific binding was observed in a range of peripheral tissues including heart, kidneys, spleen, pancreas, adrenals, lungs and liver (Noyer et al., 1995). Levetiracetam was found to bind to hippocampal membranes with modest affinity ($K_d = 780 \pm 115$ nM) and with a high binding capacity ($B_{\text{max}} = 9.1 \pm 1.2$ pmol/mg protein). Similar $K_d$ and $B_{\text{max}}$ values were obtained with the cortex, cerebellum and striatum (Noyer et al., 1995).

Clinical experience with levetiracetam is very limited. However, from the few clinical data which are available, it seems that the drug does not produce significant changes in cognitive function in patients with chronic epilepsy (Neyens et al., 1995), but significantly reduces the incidence of partial seizures (Chevalier et al., 1995). Levetiracetam has, however, demonstrated a broad spectrum of activity in experimental seizure models. It is effective against both threshold (Loscher et al., 1993) and maximal (Gower et al., 1992) convulsions induced by pentylenetetrazol and electroshock. The maximal electroshock seizure test is thought to be predictive of anticonvulsant efficacy against generalised tonic-clonic seizures, while the pentylenetetrazol seizure test indicates antiepileptic activity against generalised absence and/or myoclonic seizures. Levetiracetam also protects against seizures induced by amygdaloid kindling and those precipitated by bicuculline, picrotoxin and N-methyl-D-aspartate (Gower et al., 1992; Loscher et al., 1993). Intraperitoneal injections of levetiracetam (13-108 mg/kg) produced marked reductions in the severity and duration of both focal and secondary generalised seizures in amygdala kindled rats, and the duration of afterdischarges was also significantly lowered (Loscher et al., 1993). In audiogenic seizure-susceptible rats, levetiracetam (5.4-96 mg/kg) was found to dose-dependently protect against “wild running” behaviour.
(which precedes development of convulsions in this model) and tonic convulsions (Gower et al., 1995). In animals which did develop seizures, latency periods for both “wild running” and tonic convulsions were prolonged by levetiracetam.

1.5.9 Adverse effects
Tolerability data from a clinical trial evaluating a total of 29 patients suggested that the most common adverse side effect associated with levetiracetam were somnolence and asthenia, both being dose related (Chevalier et al., 1995). However, memory impairment, depression, diplopia mood and behavioural changes have also been described in individual patients (Sharief et al., 1996). These events generally resolved spontaneously or after dosage reduction.

1.6 Aims of the study
In this thesis several aspects relevant to the treatment of epilepsy were investigated. This work involved the following aims:

(i) To develop a freely behaving rat model that allowed simultaneous, serial sampling of blood and CSF, and also allowed continuous infusion of drug in the intraperitoneal cavity via an osmotic minipump, over at least a seven day period.

(ii) To then use this model for the study of the acute and chronic pharmacokinetics (blood) and neuropharmacokinetics (CSF) of CBZ and levetiracetam.

(iii) To investigate the anticonvulsant properties of CBZ and levetiracetam on seizure frequency, type and severity, in the tetanus toxin model of epilepsy at the same dosages used in the pharmacokinetic study.

(iv) To further elucidate the mechanism of action of levetiracetam, in an in vitro disinhibition hippocampal slice model of epilepsy.
Chapter 2 Methods and materials

2.1 Materials

2.1.1 Animals
Male Sprague-Dawley rats (Charles River, UK), weighing 250-350 g, were housed in groups of four for 7-14 days prior to surgery and were allowed free access to a normal laboratory diet (22 F diet, Labsure, Poole, Dorset) and water. A 12 hr light/dark cycle (light on 06:00 hr) was maintained.

The husbandry conditions were kept as identical for all experimental rats as was possible. All handling of the rats during surgery or in the laboratory animal experimentation room were carried out by the author. The rats were operated on in batches of two or four, depending on the surgical procedure, from the same litter when possible, on the same day, between 08:00 h and 18:00 h.

2.1.2 Drug preparation
The two drugs used were CBZ (Sigma, Poole, UK) and levetiracetam (UCB Pharmaceutical Sector, Chemin du Foriest, Belgium). CBZ behaves as a neutral lipophilic substance; it dissolves in ethanol, chloroform, dichloromethane, and other solvents but is virtually insoluble in water. Therefore, in order to achieve the continuous administration of a constant concentration of CBZ using the osmotic minipump, it was necessary to find a vehicle that was both compatible with the tissues or fluids at the site of administration and the minipumps interior reservoir, and also capable of dissolving a high enough concentration of CBZ to achieve a therapeutic level in the animal during its administration. It was also important that CBZ was stable in the vehicle solution used at 37 °C for the duration of the experiment.

The water solubility of CBZ can be enhanced substantially through formation of an inclusion complex with an amorphous cyclodextrin derivative (Brewster et al., 1997; Loscher et al., 1997; Loscher et al., 1995). Initially 2-hydroxypropyl-beta-
cyclodextrin (Sigma, Poole, UK) was tested to try and dissolve CBZ in a saline solution. However, it was found to be ineffective, as CBZ eventually precipitated out of solution after a couple of days. It was thought that the concentration being attempted to dissolve was just too great. A suspension, using propylene glycol was also not suitable for use with the minipump because the drug would again precipitate out of solution and therefore a constant concentration of drug would not be delivered. The manufacturer of the minipump quoted a maximum of 50% dimethylsulfoxide (DMSO; Sigma, Poole, UK) and 15% ethanol could be used in the minipump. By numerous in vitro trials, it was determined that CBZ dissolved in a solution composed of 42.5% DMSO, 42.5% propyleneglycol (Sigma, Poole, UK), and 15% ethanol, and remained in solution for the seven days at 37°C. Slight heating also facilitated the dissolving of the drug. As levetiracetam is water soluble, saline was therefore used as the vehicle of choice.

2.1.3 Minipump preparation

The Alzet osmotic minipump (2 ML1) (Charles River, Margate, Kent) is a miniature osmotic pump that continuously delivers test agents at controlled rates into laboratory animals. When implanted subcutaneously or intraperitoneally, these pumps serve as a constant source for prolonged drug delivery. While the pumping rate of each model is fixed at manufacture, the dose of agent delivered can be adjusted by varying the concentration of agent with which each pump is filled.

Figure 2.1 shows a cross section of the minipump. It can be seen that it is composed of three concentric layers- the drug reservoir, the osmotic sleeve containing the osmotic agent, and the rate controlling semi-permeable membrane. An additional component, the flow modulator, is a gauge stainless steel tube with a plastic end cap. This is inserted into the body of the osmotic pump after filling. The drug reservoir is the inner compartment of the pump. It is impermeable, blocking any exchange of material between the drug reservoir and the surrounding osmotic sleeve. Outside the reservoir wall is the osmotic sleeve, a cylinder containing a high concentration of sodium chloride. It is the difference in osmotic pressure between this compartment
and the implantation site that drives the delivery of the test solution. Water enters the sleeve along the osmotic gradient, compresses the flexible reservoir, and displaces the test solution through the flow modulator. The rate at which water enters the osmotic sleeve is regulated by water permeability of the semi-permeable membrane, its dimensions, and the osmotic pressure difference across the membrane.

Figure 2.1 Schematic diagram of the cross section of an osmotic minipump

The minipump was loaded with the drug of interest, according to the guidelines provided by the manufacturers, at room temperature (23 °C) and placed in isotonic saline at 37 °C until it was implanted into the peritoneal cavity of the rat (approximately 4 hr later). Information from the manufacturers state that the pumping
rate would not reach steady state for approximately 4 hr and that the pumping rate would operate at a constant rate until about 95% of its contents have been delivered and then the rate would fall rapidly to zero.

Sterile techniques, as recommended by the manufacturers were used, during the filling, handling and implantation of the minipump. As skin oils in large quantity could interfere with its performance, the minipump was handled with surgical gloves at all times. Before filling the pump with the test agent, the empty pump together with its flow moderator was weighed. The pump was filled using a 2 ml syringe (25 gauge diameter and 3.5 cm in length) and a blunt tipped filling tube (Charles River, Margate, Kent). The test solution was drawn into the syringe and the blunt tipped tube attached to the syringe. It was important that the syringe and attached tube was void of air bubbles. The pump was filled by holding the pump upright and inserting the filling tube through the opening at the end of the pump until it could go no further. This placed the tip of the tube near the bottom of the pump reservoir. The pump was filled slowly until the test solution appeared at the outlet, and then the tube was carefully removed. Excess test solution was removed and the flow moderator inserted until the cap was flush with the top of the pump. The insertion of the flow moderator normally displaced some of the test solution which was wiped off to ensure correct functioning. The filled pump was weighed and the difference in the weights gave the net weight of the solution. Usually the weight in mg was approximately the same as the volume in µl. If the volume was over 90% of the reservoir volume (i.e. 2 ml), the pump was considered ready for use. If not, there was normally trapped air inside the pump. If this was the case the pump needed to be refilled. The initial onset of the drug was delayed in some experiments, until approximately 48 hrs after surgery, by attaching a coiled tubing (PE 60; Charles River, Margate, Kent), 97 cm in length, to the end of the minipump. Silastic tubing was not recommended for use with Alzet pumps as it allows diffusional exchange of materials along the length of the catheter.
2.1.4 Cerebrospinal fluid (CSF) catheter

The CSF catheter comprised of an outer (o.d. 0.96) and inner (o.d. 0.71) polythene tubings (Portex Ltd, Hythe, Kent). The dimensions of both tubing were specifically chosen to allow insertion (with tweezers) of the inner polythene tubing into the outer polythene tubing after the bouton was formed, but not to allow the inner tubing to be readily withdrawn (Figure 2.2). The bouton was formed by gentle heating of the outer polythene tubing over a soldering iron. The function of the bouton was to prevent the removal of the inner tubing from the outer tubing, while allowing enough scope to change the length of the inner tubing in the cisterna magna after implantation. However, in order to prevent the animal from pulling out the inner catheter from the outer tubing (See Section 3.2), it was necessary to glue the inner tubing to the outer.

![Schematic diagram of the cerebrospinal fluid catheter](image)

Figure 2.2 Schematic diagram of the cerebrospinal fluid catheter
2.1.5 Anaesthetic

Prior to surgery the animals were weighed in order to determine the dosage of anaesthesia. The rats were anaesthetized by intraperitoneal injection of Hypnovel (Roche Products Ltd., Welwyn Garden City), Hypnorm (Janssen, Janssen-Cilag Ltd., Saunderton) and sterile water (2:2:4; 3.33ml/kg i.p), to final doses of 4.1 mg/kg midazolam HCL (Hypnovel), 8.2 mg/kg fluanesone and 0.26 mg/kg fentanyl citrate (Hypnorm). A single injection caused a reliable anaesthesia for the entire surgical procedure (1.0-1.5 hr) without depressing the respiratory or other vital systems. This dosage was found to be the minimum amount required for total anaesthesia during preliminary experiments, i.e. to completely abolish the withdrawal responses to pain elicited by squeezing paw pads or the skin over the neck with tweezers. Full anaesthesia usually occurred within 15 minutes. Prior to terminal experiments and the preparation of hippocampal slices, the rats were anaesthetized by an intraperitoneal injection of the above mixture.

The use of fentanyl-fluanisone in combination produces a condition of neuroeptanalgesia. Fentanyl is a potent short acting analgesic which on its own also produces some degree of central nervous depression. When given on its own it will induce nausea and vomiting in some species with a dose related cardiovascular and respiratory depression. It is commonly in commercial preparations used in combination with fluanisone, a neuroleptic tranquilizer. The effect of fluanisone is to counteract the nausea, vomiting, cardiovascular and respiratory depression and to potentiate the analgesia and sedation. The addition of a benzodiazepine such as midazolam (hypnovel) greatly increases the usefulness of the combination fentanyl-fluanisone. The dose of fentanyl-fluanisone is reduced for any given level of anaesthesia and it can be thus used for full surgical procedures. Midazolam also produces muscle relaxation.
2.2 Surgery and sampling

2.2.1 Surgical implantation of CSF and blood catheters

The rats were weighed and anaesthetised as described previously. Once fully anaesthetised the hair on the head, and chest was shaved and the skin surface was sterilised using an alcoholic steret. The animal was then placed in a Kopf stereotaxic frame with both ear bars set at zero and the nose piece set to +5 mm. A 1.5-2.0 cm incision was made 5 mm caudal to lambda and the external occipital crest, and the skin and the protective fibrous membrane were retracted. Two burr holes, one 3 mm lateral and 4 mm caudal to lambda, for the placement of a stainless steel anchor screw, and the second 2 mm lateral to the midline and 3 mm caudal to the external occipital crest and at an angle of about 40° for the catheter insertion, were made. Catheter implantation was achieved with the aid of a 37-G enamelled copper wire (Scientific Wire Co., London) contained within the catheter. This served to afford rigidity to the catheter and aided puncturing of the dura. With the bouton resting on the skull surface, the wire was withdrawn and the catheter sealed in place with dental acrylic (De Trey, Weybridge, Surrey; Figure 2.3). CSF was introduced into the catheter by a slight negative pressure using a syringe. The catheter was cut to approximately 2 cm, heat sealed, and the incision sutured (3/0 Mersilk; Ethicon, Bankhead Avenue, Edinburgh).

The rat was then placed on its back, and its forelegs were affixed to a surgical board with adhesive tape. This board contained an opening where the head of the rat rested, to prevent any damage to the CSF catheter. A 2.5 cm long incision in the skin was made in the midline between the collar bone and the sternal bone, and the external jugular vein was exposed by blunt dissection. After removing the tissue surrounding the vein with a pair of tweezers, three cotton threads were passed under the vein caudally, medially, and rostrally, and the caudal thread was ligated (Figure 2.4). Using a pair of fine scissors, the vein was perforated just below the medial thread and the catheter (Silastic tubing, 10 cm length, o.d. 1.09 mm, i.d. 0.6 mm, and cut at 45° angle; Dow, Corning, Midland, MI, USA) was inserted until 4 cm were (for a 300 g rat) within the vein. For a 250 g rat, the catheter was inserted until 3 cm were within
Figure 2.3 The CSF catheter placed in the cisterna magna of the rat and connected to the assembled sampling cannula.
The two other threads were used to secure the catheter within the vein, and the caudal thread was used to secure the positioning of the exteriorized portion of the catheter (Figure 2.4). A 1 cm cut was made behind the ear, a small pair of forceps was passed under the skin through the incision, the free end of the catheter was brought out behind the ear, and the incision was sutured (3/0 Mersilk; Ethicon, Bankhead Avenue, Edinburgh). Catheter patency was checked by flushing with 0.5 ml heparinized saline (250 U/ml Baxter Healthcare, Thetford, Norfolk). The blood catheter was then cut to a length of 3 cm and sealed using a piece of 1 cm tubing made from a 21G needle, and the rat’s chest was sutured. However, in order to prevent the animal from pulling out the blood catheter post surgery, the piece of needle used to seal the blood catheter was replaced with a piece of polythene tubing heat sealed at one end (See section 3.2). In order to minimize infection, polybactrin powder spray (The Wellcome Foundation Ltd., London) containing neomycin, polymyxin B, and bacitracin, was applied to all exposed areas prior to suturing. After surgery, rats were housed individually in perspex cages and allowed food and water ad libitum.

2.2.2 Minipump implantation

For a male Sprague Dawley rat, the manufacturers recommended that the minimum weight for a 2 ML osmotic minipump be 300 g. During the implantation of the minipump the animal was placed on its back, and its forelegs were affixed to a surgical board using adhesive tape. This board contained an opening where the head of the rat was placed, to prevent any damage to the CSF catheter or the recording electrode in some experiments. The hair of the abdomen was shaved and the skin surface sterilised using an alcoholic steret. A midline skin incision, 1.5 cm long, was made in the lower abdomen, posterior to the rib cage. The musculoperitoneal layer was raised up to avoid damage to the bowel and the layer directly beneath the cutaneous incision was incised. The minipump containing the drug of interest was inserted into the peritoneal cavity and each incision sutured separately, taking care
Figure 2.4 Blood catheter implantation - isolated vein and position of ligatures
to avoid perforation of the underlying bowel. Again to prevent infection polybactrin powder spray was applied to all exposed areas prior to suturing.

2.2.3 Post surgical procedures

After surgery, the rats were placed in individual cages and were continuously monitored until they had fully recovered from the anaesthetic. They were wrapped in tissue paper immediately after surgery, which reduced any heat loss, and placed on shredded tissue paper which insulated the animal from the cage floor and prevented any respiratory obstructions due to the wood chips. Full recovery from the anaesthetic was usually achieved 4-5 hours post-anaesthetic. Subsequently, the animals were checked daily and their general well being assessed. Once the animal was conscious they were allowed free access to food and water and a 12 hr light/dark cycle (light on 06:00 hr) was maintained.

After the experiment was completed the animals were usually killed humanely via an intraperitoneal injection of an overdose of anaesthetic or exposed to a rising concentration of carbon dioxide followed by dislocation of the neck. Rats were then left until rigor mortis was confirmed.

2.2.4 CSF sampling

The closed tip of the catheter was cut off and sampling of the CSF was achieved by connecting the implanted catheter to a cannula, a 23 G needle and 1 ml syringe. The cannula (Figure 2.3) consisted of 36.4 cm (i.d. 0.28 mm) polythene tubing, corresponding to a volume of 30 μl, and attached to a 15 cm (i.d. 0.58 mm) polythene tubing (Portex Ltd, Hythe, Kent). Gentle negative pressure was applied by aspirating with the syringe. If no CSF flow was obtained after two or three attempts, the tubing was withdrawn and the catheter carefully unblocked with the copper wire taking care that the length inserted was only 0.5 to 1 mm longer than the implanted catheter. Thus, damage to the brain tissue was unlikely. The copper wire was first slid in and out several times and then removed. The cannula tubing subsequently reconnected and negative pressure applied again. The cannula was disconnected from the catheter
between sampling, and the CSF samples were dispensed into 0.5 ml polypropylene tubes and kept at -70 °C until required for analysis. After the last sample was taken, the CSF catheter was closed off by melting the tip, using a soldering iron.

2.2.5 Blood sampling

Blood sampling was achieved by connecting the jugular vein catheter via a 1.5 cm stainless steel connector to a 30 cm polythene tubing (Portex Ltd, Hythe, Kent; i.d. 0.58 mm) filled with 0.5 ml heparinised saline (100 U/ml) and attached to a 1 ml syringe. Blood was slowly withdrawn until it reached the bottom of the syringe, the cannula was closed using a small clip, a new syringe was attached, the cannula was unclipped, and 50 μl of blood was withdrawn. If no blood emerged after several initial attempts, the rat was gently lifted and turned head down for 10 to 20 seconds and aspiration was tried again. Blood flow into the cannula could also be prompted by gently squeezing the chest wall, sternum to back, several times. When the blood flow was established the cannula and syringe were passed out through the hole in the lid of the cage, leaving enough of the cannula in the cage for the rat to be fully mobile, but without it being too lax for the animal to reach and chew through. The lid was closed and the rat left to recover from the physical handling. Thus, the only time the rat was physically handled was at the beginning and end of the sampling protocol.

In order to prevent the development of hypovolaemia, an equivalent volume of heparinized saline was administered after each sampling. Blood samples were collected in 0.5 ml centrifuge tubes (Fisher, Loughborough), which were then centrifuged (Abbot Laboratories, Maidenhead) for 5 min at 11,000 g to separate the cells from the serum. The serum was pipetted into a new tube (Fisher, Loughborough) and labelled as before and stored at -70°C until required for analysis.
2.3 CBZ and CBZ-E sample analysis

2.3.1 Materials

CBZ was obtained from Sigma (Poole, UK), and the internal standard 10-methoxycarbamazepine was obtained from Ciba Geigy, Pharmaceuticals, Horsham.

2.3.2 HPLC instrumentation and chromatographic conditions

Serum and CSF CBZ and CBZ-E concentrations were determined by high performance liquid chromatography (HPLC), based on modifications of the method of Elyas, et al., (1982). The liquid chromatograph used comprised a Spectra-Physics spectra system pump P4000, an autosampler AS3000, a UV2000 detector and a chromjet integrator (Spectra-Physics, Maidenhead). Chromatograms were run at ambient temperature on a Lichrospher 100 RP-8 column (250 x 4 mm I.D.) with a precolumn (4 x 4 mm I.D.; Merck, Poole). A mobile phase of acetonitrile, phosphate buffer pH 6.24 (23:77) with a flow rate of 1.9 ml/min was used. The column effluent was monitored at 214 nm with a sensitivity range of 1.0 a.u.f.s and a chart speed of 0.25 cm/min.

2.3.3 Preparation of buffer and standard solutions

A solution of 20 mM phosphate buffer (pH 6.24) was prepared using disodium hydrogen orthophosphate (Na₂HPO₄) and potassium dihydrogen orthophosphate (KH₂PO₄), methanol (15%) and HPLC grade water (Sigma, Poole).

A stock solution of CBZ (10 mM) and CBZ-E (10 mM) was prepared using methanol. The stock solution of 10-methoxycarbamazepine, the internal standard, was prepared using acetonitrile (ACN; Sigma, Poole). Calibration standards were prepared with blank human sera (Sigma, Poole) to provide concentrations of 5, 10, 25, 50, and 75 µmol/l, and 2, 4, 10, 20, and 30 µmol/l of CBZ and CBZ-E respectively. The standard curve was produced by plotting the peak area ratio of the standards:internal standard against the known drug concentration. The amount of drug in the unknown sample was calculated using the slope of the standard curve (Figure 2.5).
The working standard was prepared by pipetting 150 µl of stock solution into a 25 ml volumetric flask. The solution was made up to 25 ml using ACN. Three controls were prepared with blank human sera to provide concentrations of 8, 15 and 60 µmol/l and 5, 15 and 25 µmol/l of CBZ and CBZ-E respectively. These controls were normally run so as to bracket blood and CSF samples throughout the analytical run and were used as an indication whether the analytical run had successfully occurred.

### 2.3.4 Extraction procedure

To a 1.5 ml microcentrifuge tube, 20 µl of the sample or standard, 100 µl of ACN and 40 µl of the working internal standard were added. ACN served to precipitate proteins. Each tube was shaken for 10 s on a Vibrax-IKA shaker to ensure adequate mixing. The mixture was centrifuged for 5 min at 11,000 g, using a minicentrifuge (Abbott Laboratories, Maidenhead). The supernatant was then pipetted into a 150 µl glass insert and placed in a 1 ml vial. 5 µl of the prepared standards/samples were injected into the chromatograph. The chromatogram produced the data in a response-versus-
Figure 2.6 Typical chromatogram of CBZ and CBZ-E of a standard solution (a), a blood sample (b), and a CSF sample (c). The first peak (1) is CBZ-E, the second (2) is CBZ and the third (3) is the internal standard.

Various peaks can be seen on the chromatogram. The time required for a compound to pass through a liquid chromatogram is called the retention time, and under a given set of chromatographic conditions this time can be used to identify eluting compounds. It can be seen from figure 2.6 that there is adequate separation between CBZ-E, CBZ and the internal standard with retention times of 5, 8 and 11 minutes, respectively. Time zero indicates the time the sample was injected onto the column and the first peak to appear is the solvent front. The area of each individual peak is measured and this area is proportional to the quantity of compound passing through the column. CBZ and CBZ-E concentrations were determined by the ratio of the peak areas of each drug to the peak area of internal standard plotted against concentration of the drugs (Figure 2.5).
2.4 Levetiracetam sample analysis

2.4.1 Materials
Levetiracetam and the internal standard (ucb 17025; "-2, 2-trimethyl -5-oxo-pyrrolidineacetamide) were kindly donated by UCB Pharmaceutical Sector (Chemin du Foriest, Belgium).

2.4.2 HPLC: Instrumentation and chromatographic conditions
Levetiracetam concentrations in serum and CSF were determined by HPLC (Ratnaraj et al., 1996). The liquid chromatograph used comprised a Spectra-Physics spectrasystem pump P4000, an autosampler AS3000, a UV2000 detector, and a chromjet integrator. Chromatograms were run at 35°C on a steel cartridge column (250 x 4 mm I.D.) with a precolumn (4 x 4 mm I.D.) packed with LiChrospher 60 RP-Select B, 5μm (Merck, Poole, Dorset). A mobile phase of ACN/50 mM phosphate buffer pH 5.6 (15:85) with a flow rate of 0.8 ml/min at 1300 psi was used. The column eluent was monitored at 220 nm with a sensitivity range of 1.0 a.u.f.s and a chart speed of 0.25 cm/min. The mobile phase was filtered through a 0.45 μm Millipore filter before use (Amicon, Stonehouse).

2.4.3 Preparation of buffer and standard solutions
A solution of 50 mM phosphate buffer (pH 5.6) was prepared by mixing 950 ml of 50 mM potassium dihydrogen phosphate solution and 50 ml of a 50 mM disodium hydrogen phosphate solution to give a final pH of 5.6. A stock solution of levetiracetam (10 mM) was prepared using water. The stock solution of ucb 17025, the internal standard (1 mg/ml), was prepared using methanol. Calibration standards were prepared with blank human sera to provide concentrations of 25, 50, 100, 200, 300, 500, and 600 μmol/l. A typical standard curve of levetiracetam can be seen in Figure 2.7. The working standard was prepared in 50% ACN and phosphate buffer (50 mM, pH 5.6) mixture to give a final concentration of 217 μmol/l.
2.4.4 Extraction procedure

To a 1.5 ml microcentrifuge tube, 25 µl of sample or serum standard, 10 µl of 5 M sodium hydroxide solution and 25 µl of the working internal standard were added. After mixing the contents for 10 s using a vortex mixer, 250 µl of dichloromethane was added and mixed for a further 1 min using a Vibrax electronic shaker. The mixture was centrifuged for 5 min at 11,000 g. The aqueous layer was discarded and the solvent extract was then transferred to a clean microcentrifuge tube and evaporated to dryness at 50 °C using the Gyro Vap centrifugal evaporator (Howe, Banbury). The residue was reconstituted in 50 µl of mobile phase and 5 µl were injected into the chromatogram. Retention times for levetiracetam and the internal standard were 4 and 6 minutes, respectively (Figure 2.8). The total run time for each sample was 8 min. A very minor extraneous peak was detected at 3.8 min but it did not interfere with either the levetiracetam peak or the internal standard peak. Time zero indicates when the sample/standard was injected into the HPLC column.
Figure 2.8 Typical chromatograms of levetiracetam. The first chromatogram (a) is a standard, the second (b) is a blood sample and the third (c) is a CSF sample. The first peak (1) is levetiracetam and the second peak (2) is the internal standard.
2.5 Antiepileptic in vivo study

2.5.1 Animals
Male Sprague-Dawley rats (Harlan Olac, Bicester), weighing 250-350 g, were housed in groups of four for 7-14 days prior to surgery and were allowed free access to a normal laboratory diet (22 F diet, Labsure, Poole) and water. A 12 hr light/dark cycle (light on 06:00 hr) was maintained.

2.5.2 Tetanus toxin
Tetanus toxin was obtained, as a lyophilized powder, from Wellcome Research Laboratories (Beckenham, Kent, England.). A lysate of Clostridium tetani was salt precipitated and ion exchange purified. A flocculation assay was done at the supplier’s laboratory to measure toxin activity and these results were then converted into mouse LD$_{50}$ (mLD$_{50}$). This was reconstituted under aseptic conditions using normal saline to a stock solution of 1200 mLD$_{50}$/µl and stored at - 20 °C. Aliquots were subsequently diluted to final concentrations of 12mLD$_{50}$/µL for injection with phosphate buffered saline which contained 0.05 M NaH$_2$PO$_4$/NaPO$_4$ (pH 7.4) and 0.2% bovine serum albumin (Sigma, Poole, Dorset). The total sodium was 150 mM. Solutions of final concentration were stored, not longer than 4 weeks, at 4- 10 °C. The functional activity of the tetanus toxin in solution was checked in Dr. J. Mellanby’s laboratory (Department Experimental Psychology, Oxford University).

2.5.3 Electrodes
Bipolar recording electrodes were made from twisted Teflon coated stainless steel wire (bare wire diameter 0.125 mm, Medwire Corp, New York, USA) with the tips cut 500-750 µm apart. A third wire, used to earth the animal during EEG recording was connected to one of the anchor skull screws, inserted during surgery. The further end of the recording wires and the earthing wire were crimped to contact pins (Cannon Centi Loc contacts obtained from AWP Electronics Ltd, Redhill, Surrey) and later housed in Cannon Centi-Loc strip connector (AWP Electronics Ltd, Redhill, Surrey).
2.5.4 Recording apparatus
The EEG of a freely moving rat was recorded with the animal in a perspex recording cage. The headstage contacts were connected via a slip ring (Figure 2.9), which prevented the rat from tangling its recording leads, to a digitimer and the signal was then passed through a Neurolog System which consisted of an AC preamplifier, an AC/DC amplifier and finally a filter. The amplified EEG signal was digitized (1401 plus, Cambridge Electronic Design, Cambridge, UK) and stored on computer for analysis using the SPIKE2 software package (Cambridge Electronic Design, Cambridge). The EEG was monitored on-line using a digital storage oscilloscope (Philips PM3335). The animals were filmed continuously during an EEG recording session using a Panasonic infra-red camera and a Panasonic video recorder with a time lapse facility so that seizures could be reviewed and categorized. The animals were observed and checked frequently during the recording period.

2.5.5 Implantation of recording electrodes
The rats were anaesthetised with a mixture of hypnorm/hypnovel and water (2:2:4), 3.33 ml/kg i.p, as previously described. The hair on the head was clipped and the skin surface was sterilised using an alcoholic steret. The animal was then placed in a Kopf stereotaxic frame with both ear bars set at zero and the nose piece set to +5 mm. A 1.5-2.0 cm incision was made 5 mm caudal to lambda and the external occipital crest, and the skin and the protective membrane were retracted. Burr holes were drilled at three sites, one 3.5 mm lateral and 3.0 mm posterior to bregma, the second 2.0 mm lateral to the midline and 3.0 mm caudal to the external occipital crest, both for the placement of stainless steel anchor screws, and the third 3.1 mm posterior and 2.9 mm lateral to bregma for electrode implantation.

Tetanus toxin (12 mLD50) dissolved in 1 µl buffered saline, or 1 µl phosphate buffered saline control was injected manually (Hamilton 7101N syringe) over 1 minute, into the left hippocampus: coordinates 3.0 mm posterior, 3.5 mm lateral and 3.3 mm below the neocortical surface. The injection needle was left in place for a further 5 minutes to prevent toxin tracking back up the needle track. After injection
To recording equipment

Slip Ring

Cannon strip connector housing bipolar electrode

Dental cement

Skull screw

Figure 2.9 The bipolar electrode for EEG recording, housed in a cannon strip connector, placed in the hippocampus of the rat brain, and connected to the slip ring. The slip ring allowed the rat free movement around its cage.
of the tetanus toxin, a bipolar electrode was placed in the CA1 pyramidal cell layer of the right hippocampus; the coordinates of which were: 3.1 mm posterior, 2.9 mm lateral and 2.5 mm vertical. The animal was earthed by attaching a teflon coated stainless steel wire from the headstage to one of the stainless steel skull screws. Dental cement was used to form a headstage which protected the recording contact points and the animals head was sutured (Figure 2.9). Post-operative animals were housed separately with free access to food and water and allowed 24 to 48 hrs to recover. Polybactrin powder spray was applied to the wound edge, and to any exposed areas, to ensure infection did not track under the headstage.

The depth of the electrode placement was initially determined via non recovery dye injection or electrode insertion experiments, followed by hippocampus slicing using a vibroslice (Campden Instruments, Loughborough). The slices (10 μm) were then examined under the microscope. Traces of blood around the electrode shaft reduced the number of serial sections that needed to be cut and stained as the track could be identified in the block. The electrode track position was confirmed with low power (x 40) microscopy. Sections were mounted on microscope slides and stained with cresyl fast violet and haematoxylin and eosin (Figure 2.10).

Figure 2.10 Diagram showing the electrode track position, indicated by the arrow, at the CA1 pyramidal cell layer of the hippocampus
2.5.6 Recording protocol

After recovery from surgery, the rats were handled gently to familiarise them with the process prior to recording and the recording bin. EEG recording was begun, prior to the development of spontaneous epileptiform discharges. The animals were gently restrained in a tea towel for connection of the recording apparatus and then returned to the recording bin. The EEG was recorded continuously, and stored on computer for analysis using the SPIKE2 software package (Cambridge Electronic Design, Milton Rd., Cambridge). The EEG was also monitored on-line using a digital storage oscilloscope. The animals were filmed continuously during an EEG recording session using a Panasonic infra-red camera and a Panasonic video recorder with a time lapse facility so that seizures could be reviewed and categorized. Animals were housed separately during the recording session with free access to food and water. A 12 hr light/dark cycle (light on 06:00 hr) was maintained and the animals were observed and checked frequently during the recording period.

Two groups of animals were used to investigate the effect of either carbamazepine or levetiracetam on seizure type, frequency and severity. One was referred to as the control group and the other was designated the drug group. All experiments involved the injection of tetanus toxin, electrode implantation and implantation of the minipump. The control group underwent the same experimental protocol as the drug group except that the minipump contained the vehicle only.

Initial EEG recording began 1-2 days after the injection of tetanus toxin and electrode implantation, and continued for a further 5-7 days. Then the minipump containing the drug or vehicle was implanted into the peritoneal cavity of the animal, as previously described. The 5-7 days of continuous EEG recordings and continuous filming served as confirmation data of spontaneous epileptic seizures for each animal group. Following the implantation of the minipump the animals were allowed to fully recover (6-8 hrs), before they were re-connected to the recording apparatus. Continuous EEG recording and filming of the animals was undertaken for seven consecutive days which represented the life span of the minipump. The minipump was
only implanted upon confirmation of seizure occurrence. A control animal was always investigated in parallel with a drug treated animal. The control animal allowed firstly to determine the overall pattern of the syndrome and fit frequency, and secondly to ensure that the second anaesthesia and surgical procedure on the animal had no affect the overall pattern of the syndrome or seizure frequency.
2.6 Antiepileptic in vitro study

2.6.1 Animals
Male Sprague-Dawley rats (Harlan Olac, Bicester), weighing 250-300 g, were housed in groups of four prior to surgery and were allowed free access to a normal laboratory diet (22 F diet, Labsure, Poole) and water. A 12 hr light/dark cycle (light on 06:00 hr) was maintained.

2.6.2 Hippocampal slice preparation
The rats were anaesthetised by intraperitoneal injection of a mixture of hypnorm/hypnovel and sterile water as previously described, and killed by cervical dislocation. The skull was opened and the brain was rapidly removed from the skull after a cut through the cerebellum and the brain stem. The brain was glued upside down, for the preparation of horizontal slices of the ventral hippocampus on to the stage of a Vibroslice (Campden Instruments, Loughborough). The glued brain was then immersed in ice-cold oxygenated modified artificial cerebrospinal fluid (ACSF, see below) and slices cut to a thickness of 450 μm. Such slices allows for sufficient oxygen to diffuse in while being thick enough to preserve neurons and many of their local connections. Slices were then transferred to an interface recording chamber and maintained at 32-35 °C. The slices were kept in contact with a continuously perfused, warm oxygenated ACSF.

2.6.3 Artificial cerebrospinal fluid
The standard ACSF used for the electrophysiology contained the following ions: 135 mM NaCl, 16 mM NaHCO₃, 3 mM KCl, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 1 mM MgCl₂ and 10 mM glucose, equilibrated with 95% O₂-5% CO₂ gas mixture, resulting in a pH of 7.4. Warm, water saturated 95% O₂-5% CO₂ gas mixture was directed over the surface of the slices to keep the slices moist and oxygenated. Epileptic discharges were produced using bicuculline methiodide (BMI, 15 μM) (Sigma, Poole) and potassium ([K⁺]₀) raised to 5 mM.

A more protective iced cooled and freshly oxygenated ACSF was used as the solution
for the preparation of the slices. This modified ACSF was exactly as the standard ACSF except that it contained a higher concentration of MgCl₂ (3mM). The combination of low dissection temperature and elevated MgCl₂ concentration served to minimise excitotoxic damage during slice preparation.

2.6.4 Recording electrodes

Conventional microelectrode recording methods were used. Glass micropipettes for extracellular recording were filled with 3 M NaCl and had resistances of 5-20 MΩ. They were pulled on a Brown Flaming puller. An extracellular electrode was placed in the CA1 pyramidal cell layer of the hippocampus. Stimuli for evoking epileptic discharges were delivered not faster than once every 20 minutes and varied between 60-100 V amplitude and were 0.2 ms in duration. The stimulus voltage and direction were controlled by an isolated stimulator (Digitimer Ltd., Welwyn Garden City). The stimulating electrode (silver wire) was placed in the slice, activating mainly the perforant pathway.

2.6.5 Recording techniques

The recording system included an Axoprobe amplifier (Axon instruments, Burlington, CA, USA), Digitimer amplifiers and filters (Digitimer Ltd., Welwyn Garden City) and a CED 1401 (MSDOS) computer system running sigavg and Spike 2 (Cambridge Electronic Design Ltd., Milton Rd., Cambridge).

Extracellular recordings from the CA1 commenced approximately 1 hr after the preparation of the slices. Slices were checked for normal orthodromic evoked responses. Electrical stimulations, of 0.05 ms duration, were delivered every 10-30 s via a bipolar stimulating electrode fabricated from two twisted stainless steel teflon coated wires. The stimulus intensity was increased in 5 V increments until a maximal response was elicited. The stimulus intensity was then set to that producing population spikes of approximately 80-90 % of the maximum amplitude (Figure 2.11). This value of stimulus intensity was used throughout the rest of the experiment and was delivered to the slice every 15 minutes. The slices were then bathed in bicuculline methiodide
(15 μM) and \([K^+]_o\) raised to 5 mM, and left for an additional hour for the new solutions to equilibrate. Each slice acted as its own control until the bicuculline and potassium were applied to the ACSF. The evoked responses were analysed by measuring the length of the seizure-like events recorded.

![Population spike](image)

Figure 2.11 Field potential recorded from the CA1 pyramidal cell layer following single pulse stimulation of the perforant pathway

After stable epileptiform activity had been established, levetiracetam was administered at two concentrations, 200 and 400 μmol/l. Recordings of epileptiform activity were taken for one hour following the addition of each concentration of levetiracetam. The two concentrations were added cumulatively and levetiracetam was washed out for one hour at the end of each experiment. The length of four consecutive evoked responses in the absence of drug, at each of the two concentrations, and after recovery were measured in six experiments (six slices from six rats).
Chapter 3  Optimisation of the methods

3.1 Survival rates and causes of death
A summary of the survival rates, irrespective of experimental procedure or the stage of experimentation are shown in Table 3.1. The overall mortality rate of the animals investigated was 11.3 %. The majority of these deaths were as a result of respiratory distress related to anaesthesia (53.1 %)

A more detailed summary of the survival rate and causes of death for each of the studies is presented in Table 3.2. It is evident that a higher number of animals died as a result of the blood and CSF cannulation procedure (82.4 %) than that of the tetanus toxin injection procedure (17.6 %). Death through surgical manipulations during cannula insertion was rare, as were infection, bleeding, air embolism or subarachnoid haemorrhage during and after recovery.

3.2 Catheter patencies
All blood catheters from which blood could be drawn and CSF catheters from which clear CSF could be obtained were termed patent, excluding blocked and pulled off catheters and those with persistently bloody CSF. The patencies of blood and CSF catheters are presented in Table 3.3. In general, higher patency rates were obtained with blood than with CSF catheters.

3.2.1 Blood catheter patency
There were two types of sampling experiments; acute and chronic. The acute sampling protocol required only one day of both blood and CSF sampling, after which the experiment was terminated. In contrast the chronic sampling protocol required 4 days of blood and CSF sampling over a period of seven days. It was found the patency of the catheters decreased with time. By post recovery day seven, just over half (59.5 %) of the catheters initially patent were still patent or in place (i.e the animal had not physically removed the catheter). A summary of the overall causes of blood catheter patency failure can be seen in Table 3.4.
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<th>100</th>
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<td>88.7</td>
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<td>6.0</td>
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<tr>
<td>(ii) Accidental rupture of the heart</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(iii) Infection</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(iv) Subarachnoid haemorrhage</td>
<td>3</td>
<td>2.0</td>
</tr>
<tr>
<td>(v) Haemorrhage via blood cannula</td>
<td>1</td>
<td>0.7</td>
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<tr>
<td>(vi) Air embolus via blood cannula</td>
<td>2</td>
<td>1.3</td>
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<tr>
<td>(vii) Idiopathic</td>
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Table 3.1 A summary of the survival rate and causes of death in all experiments carried out, irrespective of the stage of experimentation
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<td>(iii) Infection</td>
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</tr>
<tr>
<td>(iv) Subarachnoid haemorrhage</td>
<td>3</td>
<td>2.9</td>
</tr>
<tr>
<td>(v) Haemorrhage via blood cannula</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td>(vi) Air embolus via blood cannula</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td>(vii) Idiopathic</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>Tetanus toxin injection</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Animals investigated</td>
<td>46</td>
<td>100</td>
</tr>
<tr>
<td>Survived</td>
<td>43</td>
<td>93.5</td>
</tr>
<tr>
<td>Deaths due to:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i) Respiratory distress</td>
<td>3</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Table 3.2 A summary of the survival rate and causes of death for each type of experiment carried out, irrespective of the stage of experimentation
<table>
<thead>
<tr>
<th>Time</th>
<th>CSF catheters</th>
<th>Blood catheters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acute study</td>
<td>Chronic study</td>
</tr>
<tr>
<td></td>
<td>Number</td>
<td>% Number</td>
</tr>
<tr>
<td>After surgery</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>Catheters removed (by animal)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>On post-recovery</td>
<td>42</td>
<td>7</td>
</tr>
<tr>
<td>Day 2</td>
<td>88</td>
<td>2</td>
</tr>
<tr>
<td>Day 4</td>
<td>88</td>
<td>35</td>
</tr>
<tr>
<td>Day 6</td>
<td>83</td>
<td>33</td>
</tr>
<tr>
<td>Day 7</td>
<td>78</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 3.3: Patency of blood and CSF catheters in acute and chronic studies.
In some instances when blood catheters became blocked due to clotted blood, a higher concentration of heparin (250 U) was introduced into the catheter to try to disperse the clot but this was not always successful. In these cases the experiment had to be terminated as no blood samples could be collected.

However, the biggest problem with the blood catheter was trying to prevent the animals from pulling it out (Table 3.4). Again, the number of catheters not pulled out, on post recovery day two was considerably higher than that on day 7. The various procedures undertaken so as to minimise this problem were as follows;

1. The 1 cm tubing made from a 21-G needle, used to seal the blood catheter (see section 2.2.1) was thought to cause local irritation to the rats. Therefore, this was replaced by a 1 cm polythene tubing (o.d. 0.96 mm) heat sealed at one end;

2. The length of the blood catheter exposed to the outside was kept to a minimum and;

3. The exposed length of catheter was sewn to the skin of the animal and was also taped down with adhesive tape to prevent the rats from dislodging it. These procedures were to a varying degree successful but the problem was not entirely eliminated.

3.2.2 CSF catheter patency
Higher patency rates were obtained with blood than CSF catheters. However, the same pattern was found with the number of CSF catheters being pulled out or moved slightly. A summary of the overall causes for CSF catheter failure can be seen in Table 3.5. If the intersliding polythene tubing of the CSF catheter was found to have moved position slightly, CSF did not flow continuously but intermittently, so that sometimes a sample had to be missed. It was first thought it was the animal that was moving the catheter in an attempt to remove it but it was also discovered that
<table>
<thead>
<tr>
<th></th>
<th>Sealed using needle</th>
<th>Sealed using polythene tubing</th>
<th>Catheter stitched to skin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>%</td>
<td>Number</td>
</tr>
<tr>
<td>Total catheters inserted</td>
<td>24</td>
<td>100</td>
<td>20</td>
</tr>
<tr>
<td>Total failed catheters</td>
<td>12</td>
<td>50</td>
<td>8</td>
</tr>
<tr>
<td>(i) Blocked</td>
<td>1</td>
<td>4.2</td>
<td>1</td>
</tr>
<tr>
<td>(ii) Pulled out</td>
<td>6</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>(iii) Pulled out (fatal haemorrhage)</td>
<td>1</td>
<td>4.2</td>
<td>0</td>
</tr>
<tr>
<td>(iv) Pulled out (fatal air embolus)</td>
<td>1</td>
<td>4.2</td>
<td>1</td>
</tr>
<tr>
<td>(v) Death unrelated to catheter</td>
<td>3</td>
<td>12.4</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3.4 The overall causes for blood catheter patency failure
<table>
<thead>
<tr>
<th>Description</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total catheters inserted</td>
<td>104</td>
<td>100</td>
</tr>
<tr>
<td>Total failed catheters</td>
<td>38</td>
<td>37</td>
</tr>
<tr>
<td>(i) Blocked</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>(ii) Bloody</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(iii) Pulled out</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>(iv) Fatal subarachnoid bleed</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>(v) Death unrelated to catheter</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

Table 3.5 The overall causes for CSF catheter patency failure
sometimes the tubing got caught in the top of the cage. To prevent this from happening, the bouton of the catheter was made tighter and new cages were specifically made to prevent any contact with the top of the cage and the catheter. It was then harder for the animals to move the CSF catheter. However, this solution did not prevent the animal from dislodging the catheter entirely. In order to minimise this risk, the intersliding polythene tubing of the CSF catheter was glued to the outer tubing. This proved to be quite successful.

### 3.3 Validation of HPLC methods

The analysis of samples, standards and controls, for CBZ, CBZ-E and levetiracetam content, were carried out in duplicate. A linear relationship between the peak area ratios (drug/internal standard) and the concentrations of CBZ, CBZ-E and levetiracetam was confirmed by adding known amounts of drug to blank sera and subjecting these to the appropriate extraction procedure and chromatography. Quantitation was achieved by the peak area ratios of the drug to internal standard and these were linearly related over the range of 5-75 μmol/l, 2-30 μmol/l and 25-600 μmol/l, for CBZ, CBZ-E and levetiracetam respectively. The calibration data from six standard curves for CBZ, CBZ-E and levetiracetam fitted linear equations with correlation coefficients ranging from 0.996 to 0.999.

Within-batch precision was determined from the analysis of spiked sera at various concentrations of CBZ, CBZ-E and levetiracetam. Tables 3.6, 3.7 and 3.8 represent the within-batch and between-batch imprecision for CBZ, CBZ-E and levetiracetam, respectively, in serum standards. Overall, the CV’s were <12 % (2 to 12 %). Furthermore, accuracy was acceptable as ratios between calculated and assayed values were close to unity (mean ± SEM; 0.951 ± 0.006, 0.965 ±0.02 and 0.982 ± 0.0003 for CBZ, CBZ-E and levetiracetam, respectively, using data for between-batch imprecision).

### 3.4 Minipump implantation

By use of a minipump, a continuous dosing strategy can be achieved. However, in
<table>
<thead>
<tr>
<th>Control ( ^1 ) (( \mu \text{mol/l} ))</th>
<th>Within-batch (( \text{N}=20 ))</th>
<th>Between-batch (( \text{N}=20 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>( 5 )</td>
<td>( 5.4 ) ( \pm 0.1 )</td>
<td>( 5.3 ) ( \pm 0.1 )</td>
</tr>
<tr>
<td>( 15 )</td>
<td>( 16.6 ) ( \pm 0.3 )</td>
<td>( 15.5 ) ( \pm 0.4 )</td>
</tr>
<tr>
<td>( 40 )</td>
<td>( 42.5 ) ( \pm 0.5 )</td>
<td>( 42.6 ) ( \pm 0.6 )</td>
</tr>
<tr>
<td>( 60 )</td>
<td>( 60.4 ) ( \pm 0.8 )</td>
<td>( 63.1 ) ( \pm 0.6 )</td>
</tr>
</tbody>
</table>

\( ^1 \) Calculated value from amount of CBZ added to serum

Table 3.6 Within-batch and between-batch imprecision for the measurement of CBZ in serum by HPLC (controls were prepared by adding known amounts of CBZ to serum)
<table>
<thead>
<tr>
<th>Control $^1$ (µmol/l)</th>
<th>Within-batch (N=20)</th>
<th>Between-batch (N=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (µmol/l)</td>
<td>±SEM</td>
</tr>
<tr>
<td>5</td>
<td>4.8</td>
<td>0.1</td>
</tr>
<tr>
<td>15</td>
<td>15.7</td>
<td>0.1</td>
</tr>
<tr>
<td>25</td>
<td>25.1</td>
<td>0.2</td>
</tr>
</tbody>
</table>

$^1$ Calculated value from amount of CBZ-E added to serum

Table 3.7 Within-batch and between-batch imprecision for the measurement of CBZ-E in serum by HPLC (controls were prepared by adding known amounts of CBZ-E to serum)
<table>
<thead>
<tr>
<th>Control(^1) (µmol/l)</th>
<th>Within-batch (N=20)</th>
<th>Between-batch (N=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ±SEM CV</td>
<td>Mean ±SEM CV</td>
</tr>
<tr>
<td></td>
<td>(µmol/l) %</td>
<td>(µmol/l) %</td>
</tr>
<tr>
<td>40</td>
<td>40.1 ±0.4 4</td>
<td>40.8 ±0.5 6</td>
</tr>
<tr>
<td>100</td>
<td>101.1 ±0.9 4</td>
<td>101.1 ±1.0 4</td>
</tr>
<tr>
<td>250</td>
<td>251.1 ±0.9 2</td>
<td>255.6 ±1.2 2</td>
</tr>
</tbody>
</table>

\(^1\) Calculated value from amount of levetiracetam added to serum

Table 3.8 Within-batch and between-batch imprecision for the measurement of levetiracetam in serum by HPLC (controls were prepared by adding known amounts of levetiracetam to serum)
some instances during the chronic studies, some blood and CSF samples were found to contain no CBZ or CBZ-E. At post-mortem, it was found that the exit of the minipump had been blocked by a portion of alimentary canal and the drug had precipitated at that point. In order to prevent this from occurring subsequently, a short length of tubing (PE 40, Charles River, Margate, Kent), approximately 1.5 cm, was attached to the minipump. This proved successful in preventing the blockage of the minipump by the alimentary canal. A post mortem examination was carried out after each experiment in order to confirm that the minipump had pumped for the entire duration and that there was no drug left in the minipump after the seven days.

3.5 Electroencephalographic (EEG) recording
Initially when recordings from a bipolar electrode with no earth system was undertaken it was observed that the noise to sample recording ratio was very high. This was due to 50 Hz activity in the surrounding area and also to movement of the animal in the cage. This produced large 50 Hz oscillations and movement artifacts throughout the EEG recording, and some recordings of seizures were completely enveloped by this noise. This high noise to sample ratio also led to EEG recordings of very low amplitude. Initially, in order to eliminate this problem, earthing leads were connected to various pieces of equipment that may have been the cause of the 50 Hz activity. Eradicating the noise due to the movement of the rat was achieved by including an earthing lead into the electrode and connecting this to a skull screw placed in the skull during the surgical procedure. The skull screw was normally placed in the hole burred for the injection of the tetanus toxin. This improved the EEG recordings immensely, the amplitude of the recordings was increased 5 fold, and it also cleared the recordings of the majority of movement artifact.

3.6 Tetanus toxin model
As the number of seizures progressed during the seizure syndrome the animals became increasingly difficult to handle. They would resort to biting or leaping out of their cages in an attempt to escape. This was incredibly stressful to both the handler and the animal concerned which if continued would have affected the overall results.
Handling the animal every day in order to improve the situation did not work, in fact the animal became even more distressed.

Use of the minipump, decreased the number of times the animal needed to be injected with the drug in question, dramatically from 2-3 times a day over seven days down to once. However it was still necessary to inject the animal with the anaesthetic prior to minipump implantation. This was achieved by covering the rat with a cloth, which calmed the rat down. Once the rat was relaxed, it was quickly picked up and administered the anaesthetic.
Chapter 4 Carbamazepine kinetics

4.1 Carbazepine chronic kinetic study

4.1.1 Experimental protocol

The initial objective of the CBZ chronic study was to establish the time taken for CBZ and CBZ-E to reach steady-state concentrations in the blood and CSF. Male Sprague Dawley rats (n=5) were administered with 4 mg/kg/hr CBZ, via the osmotic minipump. The surgical procedure involved implanting a blood catheter in the jugular vein, a CSF catheter in the cisterna magna and the osmotic minipump, containing CBZ, in the intraperitoneal cavity. The initial onset of the drug was delayed until approximately 48 hrs after surgery, by attaching a coiled tubing (PE 40), 97 cm in length, to the end of the minipump. CSF (30 μl) and blood (50 μl) samples were collected at 30 min intervals over an 8-10 hour period. Samples were stored at -70 °C until they were required for analysis. Samples were analysed for CBZ and CBZ-E content by HPLC.

The second stage involved determining the concentrations of CBZ and CBZ-E in blood and CSF throughout the life-span of the minipump. Male Sprague Dawley rats were administered i.p. with 2 and 4 mg/kg/hr (n=6) CBZ, respectively, via the osmotic minipump. The surgical procedure involved implanting a blood catheter in the jugular vein, a CSF catheter in the cisterna magna and the osmotic minipump, containing CBZ, in the intraperitoneal cavity. On days 2, 4, 6, and 7 after surgery CSF (30 μl) and blood (50 μl) samples were simultaneously withdrawn every 30 min for a 6-8 hour period. Samples were stored at -70 °C until they were required for analysis of CBZ and CBZ-E content by HPLC.

4.1.2 Blood pharmacokinetics

The mean serum concentration versus time profiles of CBZ and CBZ-E (Figures 4.1 and 4.2) demonstrate rapid absorption following intraperitoneal administration (4mg/kg/hr CBZ). CBZ and CBZ-E first appeared in the blood within 0.5 and 2 hrs respectively and rose gradually to peak concentrations. Peak concentrations (Cmax)
Figure 4.1 Serum (■ # ) and CSF (— □ — ) concentration versus time profiles of CBZ, post CBZ administration (4 mg/kg/hr), over a ten hour period. Values are mean ± SEM of 6 rats.

Figure 4.2 Serum (— — ) and CSF (— □ ) concentration versus time profiles of CBZ-E, post CBZ administration (4 mg/kg/hr), over a ten hour period. Values are mean ± SEM of 6 rats.
for CBZ and CBZ-E were achieved at 3.5-7.0 hrs and 7.5-8.5 hrs respectively (Table 4.1). The mean Cmax values for CBZ and CBZ-E were 40.4 ± 2.9 and 39.6 ± 1.8 μmol/l (Table 4.1). Since both Tmax and Cmax values were determined from the tables, and the first time point was at 30 minutes, these values may have been overestimated and underestimated respectively. As CBZ was administered continuously during the period of sampling, the mean maximum (Cpss max; 28.4±1.5, 38.3±1.3), mean minimum (Cpss min; 23.9±1.9, 31.3±1.3) and mean average (Cpss ave; 26.2±1.4, 34.8±1.1) concentrations in the blood were calculated for CBZ and CBZ-E respectively, once steady-state concentrations were achieved (Table 4.2).

Post administration of the higher dose of CBZ, steady-state concentrations of CBZ and CBZ-E were achieved at 3.5 and 7 hours respectively (Figures 4.1 and 4.2), and the mean blood to CSF ratio of CBZ was 3.88 ± 0.5. As was expected higher concentrations of both CBZ and CBZ-E were observed in blood than in CSF. At steady state concentrations variation was observed in CBZ concentrations determined in the blood (range; 20.9-33.09 μmol/l; Figure 4.1, 4.2). This variation was, however, less prominent in blood CBZ-E concentrations (range; 31.9-39.8 μmol/l; Figure 4.1, 4.2).

During the chronic study, by the second day of sampling, CBZ concentrations in the blood compartment were found to be significantly less than those of the previous day (Figures 4.5 and 4.6). Thereafter there was a gradual decline in blood concentrations over the following 5 days. Similarly a significant fall in blood CBZ-E concentrations was observed, but this occurred later at day 2-4 (Figures 4.3, 4.4, 4.5 and 4.6). This reduction in concentrations was, however, independent of dose, i.e. the time at which blood CBZ and CBZ-E concentrations fell, differed between the doses. CBZ and CBZ-E concentrations both fell between day 4 and 6 respectively, with the lower dose used.

Finally during the initial study, significantly higher concentrations of the metabolite
<table>
<thead>
<tr>
<th>Dose</th>
<th>Rat</th>
<th>Tmax (h)</th>
<th>Cmax (μmol/l)</th>
<th>Tmax (h)</th>
<th>Cmax (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mg/kg/h</td>
<td>1</td>
<td>7.0</td>
<td>49.9</td>
<td>8.5</td>
<td>46.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.5</td>
<td>43.9</td>
<td>7.5</td>
<td>37.0</td>
</tr>
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<td></td>
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<td>35.2</td>
<td>8.5</td>
<td>36.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.0</td>
<td>34.8</td>
<td>8.5</td>
<td>38.7</td>
</tr>
<tr>
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<td>5</td>
<td>3.5</td>
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<td>8.3</td>
<td>39.6</td>
</tr>
<tr>
<td>±SEM</td>
<td></td>
<td>0.8</td>
<td>2.9</td>
<td>0.2</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 4.1 Serum pharmacokinetic constants of CBZ and CBZ-E, following intraperitoneal administration of CBZ
<table>
<thead>
<tr>
<th></th>
<th>CBZ</th>
<th>CBZ-E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood ±SEM (µmol/l)</td>
<td>CSF ±SEM (µmol/l)</td>
</tr>
<tr>
<td>Cpps max</td>
<td>28.4 ± 1.5</td>
<td>8.5 ± 1.7</td>
</tr>
<tr>
<td>Cpps min</td>
<td>23.9 ± 1.9</td>
<td>5.6 ± 1.5</td>
</tr>
<tr>
<td>Cpps ave</td>
<td>26.2 ± 1.4</td>
<td>7.1 ± 1.3</td>
</tr>
</tbody>
</table>

Table 4.2 The mean maximum, minimum and average concentrations in blood and CSF of CBZ and CBZ-E
Figure 4.3  Serum (---) and CSF (---) concentration versus time profiles of CBZ, post CBZ administration (2mg/kg/hr), over a seven day period. Values are mean ± SEM of 6 rats.
Figure 4.4 Serum (...) and CSF (■■) concentration versus time profiles of CBZ-E, post CBZ administration (2mg/kg/hr), over a seven day period. Values are mean ± SEM of 6 rats.
Figure 4.5 Serum (○) and CSF (□) concentration versus time profiles of CBZ, post CBZ administration (4mg/kg/hr), over a seven day period. Values are mean ± SEM of 6 rats.
Figure 4.6  Serum (●) and CSF (□) concentration versus time profiles of CBZ-E, post CBZ administration (4mg/kg/hr), over a seven day period. Values are mean ± SEM of 6 rats.
than of the parent drug were achieved 5-6 hours post drug administration, and this pattern continued during the period of the chronic study. Furthermore, during the chronic study, following administration of the lower dose, the concentration of CBZ-E was approximately double that of CBZ. Whereas, with the higher dosing regimen, the concentration of CBZ-E was approximately three times that of CBZ.

4.1.3 CSF neuropharmacokinetics

Figures 4.1 and 4.2 show respectively the CSF CBZ and CBZ-E concentration versus time profiles, following i.p. administration of CBZ (4 mg/kg/hr). Whilst CBZ was detected simultaneously in both blood and CSF compartments at 0.5 h post CBZ administration, CBZ-E was not detectable until the sixth time point, 150 minutes post-dose (Figure 4.2). Thereafter, concentrations of both CBZ and CBZ-E rose gradually with peak concentrations achieved at 3.5-9.0 hrs and 7.0-9.0 hrs respectively (Table 4.3). The mean Cmax values for CBZ and CBZ-E were respectively 11.8 ± 1.3 and 22.7 ± 1.0 µmol/l (Table 4.3).

Although the concentration versus time profiles of CBZ and CBZ-E in the CSF paralleled that of the blood compartment, steady-state concentrations were achieved somewhat later at 6 hrs post drug administration. In addition, the rise in CBZ and CBZ-E concentrations was much more gradual than that observed in the blood. The same variation observed in CBZ blood concentrations was also observed for CSF, but was less marked. In contrast CSF CBZ-E concentrations did not exhibit any significant variation. Cpss max (8.5±1.7, 18.8±1.7), Cpss min (5.6±1.5, 16.9±1.3) and Cpss ave (7.1±1.3, 17.9±1.1) CBZ and CBZ-E concentrations in CSF, respectively, were determined once steady-state was reached (Table 4.2). Additionally, similar to that found in the blood, higher CBZ-E than CBZ concentrations were detected in the CSF compartment.

The mean CSF/serum concentration ratio, for CBZ and CBZ-E, rises to approximately steady-state value by 5.5 hrs and 6.0 hrs, respectively (Figure 4.7). There was however, a large inter-rat variability and the range varied from 3.0-5.5 hrs.
<table>
<thead>
<tr>
<th>Dose</th>
<th>Rat</th>
<th>Tmax (h)</th>
<th>Cmax (µmol/l)</th>
<th>Rat</th>
<th>Tmax (h)</th>
<th>Cmax (µmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mg/kg/h</td>
<td>1</td>
<td>5.0</td>
<td>11.0</td>
<td>2</td>
<td>3.5</td>
<td>16.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3.5</td>
<td>16.1</td>
<td>3</td>
<td>9.0</td>
<td>12.8</td>
</tr>
<tr>
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<td>4</td>
<td>5.0</td>
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<td>5</td>
<td>8.5</td>
<td>10.6</td>
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<tr>
<td>Mean</td>
<td></td>
<td>6.2</td>
<td>11.8</td>
<td>8.5</td>
<td>8.5</td>
<td>22.7</td>
</tr>
<tr>
<td>±SEM</td>
<td></td>
<td>1.1</td>
<td>1.3</td>
<td>0.4</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3 CSF neuropharmacokinetic constants of CBZ and CBZ-E, following intraperitoneal administration of CBZ
Figure 4.7 CSF/Serum concentration ratio, for CBZ (---) and CBZ-E (----)
and 3.0-7.0 hrs, respectively. At steady-state concentrations the mean CSF to serum ratio of both CBZ and CBZ-E was 0.34 ± 0.03 and 0.54 ± 0.02, respectively. Finally, following continuous administration of CBZ, CSF CBZ and CBZ-E concentration versus time profiles paralleled those observed in the blood compartment. Thus, after steady-state concentrations of CBZ and CBZ-E were achieved there was a gradual decline in concentrations over the subsequent 5 days (Figures 4.3, 4.4, 4.5 and 4.6). The degree of decline in concentration was dependent on dose, as a more significant fall was observed with the higher dose.

4.1.4 Discussion

The rat model used for this investigation and subsequent pharmacokinetic and neuropharmacokinetic studies was chosen so that: interindividual variability (as occurs when composite values of individual rats killed at different time points after drug administration are used) was minimised and thus the number of experimental animals needed to obtain detailed kinetic data was reduced, and long sampling protocols could be used.

Although, as part of the development of any AED, many studies are undertaken to determine its blood pharmacokinetics in a variety of species, there is a sparcity of published data on AEDs being administered continuously via the Alzet osmotic minipump (Loscher, 1986; Ohda et al., 1996). In fact, to date, this is the first study which reports on the use of the minipump to deliver CBZ in a pharmacokinetic animal model. Most of the previous work with CBZ, has involved its administration via injection intravenously or intraperitoneally. Generally a single injection into the peritoneal cavity normally results in a rapid appearance of drug in blood followed by a rapid rise in concentration and finally concentrations fall exponentially. Whereas, continuous infusion, as occurs by use of the minipump, accompanied by peak and trough plasma concentration fluctuations results in steady-state concentrations.

The serum and CSF concentration versus time profiles of CBZ and CBZ-E, exhibited biphasic characteristics: the first phase lasted approximately 2 days and was
associated with a gradual increase in CBZ and CBZ-E concentrations until maximum concentrations were achieved. Henceforth, these concentrations will be referred to as ‘steady-state’ concentrations. CBZ and CBZ-E rapidly appeared in serum and CSF compartments (0.5 h and 2 h, respectively) suggesting ready penetration from the peritoneal cavity to serum, and from serum to brain and finally the CSF compartment. The dose-dependent increase in both serum and CSF CBZ and CBZ-E after CBZ administration (2 and 4 mg/kg/h) suggests that transport across the blood brain barrier is not rate limiting over the concentration range observed in the present study. The second phase was associated with a significant reduction in serum and CSF CBZ and CBZ-E concentrations and occurred between days 2 and 4 post CBZ dosage initiation. Subsequently concentrations exhibited oscillatory characteristics.

At steady-state, one would expect the concentrations to remain relatively constant (ie the rate of administration to equal the rate of elimination). However, in the present study, with both dosing regimens, post steady-state concentrations, there was a significant reduction followed by a decreasing trend in serum and CSF CBZ and CBZ-E concentrations throughout the remainder of the chronic study period (Figures 4.3, 4.4, 4.5 and 4.6). This decrease, however, was not due to the drug precipitating out of solution within the minipump, as was determined by post mortems carried out, where the minipump was inspected and no precipitation was found. Indeed, there is evidence that this reduction can be associated with CBZ's ability to induce its own metabolism. This is referred to as autoinduction. The main metabolic pathway of carbamazepine is epoxidation to carbamazepine-10,11-epoxide (CBZ-E), followed by conversion to trans-10,11-dihydro-10,11-dihydroxycarbamazepine (trans-diol II), as demonstrated below;

\[
P450 \text{ enzyme} \rightarrow \text{hydrolase} \rightarrow \text{Carbamazepine} \rightarrow \text{Carbamazepine-10,11-epoxide} \rightarrow \text{trans-diol.}
\]

The initial reaction is catalysed by hepatic enzymes of the microsomal cytochrome P-450 (Van Boxtel et al., 1981). They are located mainly in the endoplasmic
reticulum (Nebert et al., 1991, Ryan & Levin, 1990, Guengerich, 1992, Gonzalez, 1988). This family of isozymes is known to be strongly induced by repeated administration of CBZ in both humans and animals (Regnaud et al., 1991). Induction is associated with an increased liver weight, increased total hepatic cytochrome P-450 concentration, and increased NADPH-cytochrome reductase, aminopyrine N-demethylase, and UDP-glucoronyltransferase activities (Wagner et al., 1987). The cytochrome P-450 isoforms responsible for metabolism of this drug are cytochrome P-4502B1 and P-4502B2. Cytochrome P-4503A levels are also increased slightly, following continuous treatment with CBZ (Panesar et al., 1996). Induction results in an acceleration of metabolism, followed by a consequent attenuation in blood and CSF CBZ concentrations.

Moreover, CBZ-E is almost completely converted to the trans-diol metabolite by the enzyme epoxide hydrolase. This enzyme is also inducible by repeated doses of CBZ, although to a lesser extent than the P-450 isozymes (Patel et al., 1978; Tybring et al., 1981). Epoxide hydrolase is located in the microsomal fraction of the liver cell (Tybring et al., 1981) and its induction may be also partly responsible for the decline in concentrations of CBZ and its metabolite observed in this thesis. Additionally, these data are in agreement with other studies that have shown that repeated CBZ administration leads to a marked and significant increase in the clearance of the drug and a decrease in steady-state concentrations in numerous species including the rat (Farghali-Hassan et al., 1976; Morselli et al., 1977; Pitlick & Levy, 1977; Patel et al., 1978; Wedlund et al., 1983; Frey & Loscher, 1985).

However, it is somewhat difficult to directly compare the results presented in this thesis with previous investigations, as many of the experimental conditions were different. Such differences included; the mode of administration, the composition of the vehicle, the sampling time period, the dosing regimen and finally a freely mobile/moving rat model being used. Nevertheless, some of the data are comparable. For example, in the present study higher concentrations of CBZ-E compared to CBZ, were detected, 5-6 hours post CBZ administration and remained higher for the
remainder of the sampling period. This is in good agreement with previous studies where it was demonstrated that the epoxide metabolite had a longer elimination half-life than CBZ itself (Faigle & Feldmann, 1977; Frey & Loscher, 1980). For instance in the dog, the apparent plasma half-life of CBZ following a single dose is 1.1 to 1.9 hrs. Whereby the apparent plasma half-life for CBZ-E was estimated to be 1.6 to 7.0 hr (Frey & Loscher, 1985). Furthermore, in the rat, following intravenous administration of CBZ, the peak CBZ-E concentrations occurred 1-2 hr and then declined more slowly than CBZ, again suggesting a longer half-life of CBZ-E compared to CBZ (Remmel et al., 1990). In addition, the preferential induction by CBZ of epoxide hydrolase (mentioned previously) may also be contributing to the higher plasma concentrations of CBZ-E.

Indeed, the fact that higher concentrations of serum CBZ-E than CBZ are found in the rat is quite interesting. This is complete contrast to that observed in man. CBZ-E concentrations in human plasma range usually between 10-20 % in monotherapy and 20-50 % in some polytherapy combinations, of that of CBZ (Bertilsson et al., 1986). In addition the half life of CBZ-E of 6 hours, in non-induced humans, is considerably shorter than that of CBZ (36 hours). These contrasts demonstrate the limitations of using animal pharmacokinetic models and that caution must be used when the comparability of in vivo results for the clinical situation have to be estimated.

The concentration of CBZ-E in the blood was approximately twice that of CBZ, following the lower dose administration. This factor increased to approximately three times, following administration of the highest dose. This supports earlier work carried out by Remmel et al. (1990) who observed that as the clearance of CBZ decreased with larger doses, a greater proportion of the dose was metabolised to the epoxide and that the formation clearance of CBZ-E was not changed by increasing dose (Remmel et al., 1990). Because autoinduction is both time and dose dependent it would be expected that with higher dosing regimens autoinduction would be greater than that of the lower dosages.
The variation observed in CBZ concentrations in blood and CSF is typical of CBZ (Figure 4.1 and 4.2). Circadian oscillations in plasma CBZ concentrations have been previously reported in the rhesus monkey administered CBZ by constant infusion (Levy et al., 1984; Wedlund et al., 1983). According to the authors, the phenomenon is not due to variations in metabolic activity, but may be linked to the circadian rhythmicity of several physiological processes (e.g. plasma protein concentrations, haemodynamics, day/night changes, motor activity). These oscillations can be clearly seen in the CBZ blood concentration versus time profile during the first day of sampling but were less apparent in the CSF, and less pronounced for CBZ-E (Figure 4.1 and 4.2).

It may be possible that certain components of the vehicle used during this study had an effect in some way on the kinetics of the drug. For instance, there is evidence that in animals, absorption and tissue distribution of CBZ is influenced to a certain extent by the vehicle employed (Morselli et al., 1971). This is an important point which is seldom taken into consideration as it may play a certain role in determining the drugs effect. Ethanol is known to have an inducing effect on the cytochrome P450 metabolising enzymes. Thus, the ethanol of the vehicle used in this study, could have had an impact on the concentration profiles of CBZ and CBZ-E. However, since only approximately 43 µl of ethanol was delivered to the rat per day, it was unlikely that ethanol had a significant effect on the respective enzymes. However, this assumption was not experimentally investigated.

Because intraperitoneal administration was used in this thesis and not intravenous administration, accurate determination of rates of entry into the CSF compartment are not possible. Nonetheless, equilibration between the CSF and blood compartments could be readily determined and occurred within 6 hours for both CBZ and CBZ-E, with the higher dosing regimen. The delayed appearance of CBZ-E in the blood and CSF compartments reflects the time required to convert CBZ into its metabolite CBZ-E.
The issue of dead volume in the CSF and blood catheters needs to be considered. With regards to the CSF there was no dead volume as the catheter was not flushed. Instead, a volume of CSF was always present in the catheter which represented the previous sampling time and this disparity was adjusted for in the pharmacokinetic analysis. With regards to the blood catheter, flushing using a heparinised saline solution was undertaken. However, during sampling this solution, which comprised the dead volume of the catheter, was not used for the analysis of drug content. Hence the dead volume of the blood catheter did not impact on the pharmacokinetic data.

In conclusion, CBZ and CBZ-E showed very complicated pharmacokinetics. Firstly, CBZ rapidly appeared in both serum and CSF compartments, following its initial administration. Secondly, steady-state concentrations were reached by 4-5 hours, post CBZ administration. Subsequently, autoinduction was evident, as revealed by the marked decrease in blood and CSF CBZ concentrations. Accelerated CBZ metabolism began on the second day of dosing, after which there was a gradual decline in concentrations during the remaining 4-5 days of sampling. In contrast, CBZ-E was not detected during the first couple of hours of sampling and autoinduction of CBZ-E metabolism began later at 2-4 days, than that of CBZ, post CBZ administration. Finally by approximately 5 hrs after initiation of CBZ administration, higher concentrations of CBZ-E, compared to CBZ were achieved and this pertained for the remainder of the study period. These unique pharmacokinetic properties of CBZ which are widely known and are well documented, make CBZs clinical use somewhat difficult. However, as complicated pharmacokinetics and undesirable side effects are associated with the majority of the older AEDs that are currently in use for the treatment of epilepsy, there is a growing need for new AEDs with desirable pharmacokinetic properties and improved risk/benefit ratios.
Chapter 5  Levetiracetam Kinetics

5.1  Levetiracetam acute kinetic study

5.1.1  Experimental protocol

The aims of this study were to determine the pharmacokinetics (blood) and neuropharmacokinetics (CSF) of levetiracetam in individual rats by repetitive withdrawal of blood and CSF fluid. Male Sprague Dawley rats were implanted with blood and CSF catheters, and allowed 48 hrs to recover at which point they were administered with 20, 40 or 80 mg/kg (n=6) levetiracetam via injection in the intraperitoneal cavity. CSF (30 µl) and blood (50 µl) samples were collected at 15 min intervals for the first 30 minutes, and then at 30 min intervals for a further 8-10 hours. The CSF and total and free concentrations of levetiracetam in serum were determined by HPLC. Samples were stored at -70 °C until they were required for analysis.

5.1.2  Blood pharmacokinetics

Figure 5.1 shows the serum concentration versus time profiles of levetiracetam following intraperitoneal injection of levetiracetam (20, 40 and 80 mg/kg). Results are given as mean ± SEM for 6 rats. Levetiracetam concentration rose essentially linearly and dose-dependently (Figure 5.2) with mean peak concentrations achieved at 0.25- 0.5 hr (Figure 5.1). Concentrations then fell exponentially. A total of 18 random sera, chosen to reflect the spectrum of sampling time and levetiracetam dose, were analysed for content of free non-protein-bound levetiracetam. The free/total serum levetiracetam ratio varied between 0.90-1.07 (mean±sem, 1.01±0.02) and was not time- or concentration-dependent (Figure 5.3).

Table 5.1 shows the apparent pharmacokinetic constants for individual rats together with the mean values, as calculated from log concentration versus time plots. The pharmacokinetic constants for individual rats showed moderate variability within the three dose groups with values varying over a 1-2.3 fold range. Mean Cmax values for levetiracetam increased proportionately from 20 to 40 mg/kg but these values for 80
Figure 5.1: Serum leviracetam concentration versus time profiles, post i.p. administration of leviracetam 20 mg/kg (---), 40 mg/kg (---) and 80 mg/kg (. - . -). Values are mean ± SEM of 6 rats.
Figure 5.2 AUC and Cmax versus levetiracetam dose profiles for serum and CSF. Correlation co-efficients for a, b, c and d are 0.933, 0.978, 0.943 and 0.958 respectively.
Figure 5.3 Serum levetiracetam free/total concentration ratio at different time periods demonstrating that levetiracetam was not protein bound.
<table>
<thead>
<tr>
<th>Rat No</th>
<th>Tmax (h)</th>
<th>Cmax (µmol/l)</th>
<th>AUC (µmol/l/hr)</th>
<th>t_{1/2} (h)</th>
</tr>
</thead>
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<tr>
<td>20mg/kg</td>
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<td>559</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>166</td>
<td>648</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
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<td>156</td>
<td>624</td>
<td>2.5</td>
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<td></td>
<td>0.25</td>
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<td>352</td>
<td>1.9</td>
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<tr>
<td></td>
<td>0.25</td>
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<td>433</td>
<td>2.1</td>
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<td></td>
<td>0.25</td>
<td>102</td>
<td>330</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Mean ±sem 0.25 134 491 2.2

40mg/kg

|        | 0.25 | 268 | 799 | 1.9 |
|        | 0.50 | 300 | 748 | 1.6 |
|        | 0.50 | 234 | 816 | 2.2 |
|        | 0.25 | 278 | 750 | 1.6 |
|        | 0.25 | 159 | 479 | 1.9 |
|        | 0.50 | 180 | 558 | 1.9 |

Mean ±sem 0.37 237 692 1.8

80mg/kg

|        | 0.50 | 650 | 2845 | 3.2 |
|        | 0.50 | 608 | 2704 | 2.3 |
|        | 0.50 | 688 | 2438 | 3.0 |
|        | 0.50 | 636 | 2572 | 2.8 |
|        | 0.50 | 649 | 2685 | 2.9 |
|        | 0.50 | 679 | 2442 | 2.5 |

Mean ±sem 0.50 652 2664 2.8

Table 5.1 Pharmacokinetic constants for blood (serum) after intraperitoneal administration of levetiracetam (20, 40 and 80 mg/kg). Tmax = time to maximum concentration; Cmax = maximum concentration; AUC_{(0-8hr)} = area under the concentration versus time curve; t_{1/2} = apparent elimination half-life.
mg/kg were slightly higher than twice those achieved for 40 mg/kg. Mean t1/2 values were indistinguishable for the groups given 20 and 40 mg/kg with values varying between 1.8-2.2 hr. A slightly higher mean t1/2 value (2.8 hr) was observed in the group given 80 mg/kg levetiracetam. This difference was also reflected in the higher mean AUC value in the latter group.

5.1.3 CSF neuropharmacokinetics

Figure 5.4 shows the corresponding CSF concentration versus time profiles of levetiracetam. Levetiracetam was detectable in the CSF at the first time point, 15 minutes post dose and concentrations rose dose-dependently (Figure 5.2). For all three doses the concentration then rose over a period of 1.0-2.0 h up to a maximum, and then slowly declined. The calculated neuropharmacokinetic constants for individual rats together with the mean values, as determined from log concentration versus time plots can be seen in table 5.2. The neuropharmacokinetic constants for individual rats showed moderate variability within the three dose groups with values varying over a 1-3 fold range. CSF levetiracetam concentrations peaked somewhat later (mean Tmax, 1.33-1.91 hr) than blood levetiracetam. Mean Cmax values for CSF levetiracetam were slightly lower to those in serum with the CSF/serum ratio independent of dose (0.8, 0.7, and 0.8 at 20, 40 and 80 mg/kg, respectively). Although CSF mean t1/2 values were dose-independent, as seen in serum, values were significantly larger for every levetiracetam dose compared to values observed for serum.

Figure 5.5 shows the levetiracetam CSF/serum concentration ratio over time for 20, 40 and 80 mg/kg levetiracetam. It can be seen that equilibration between the blood and CSF compartments (as measured by a constant CSF/serum levetiracetam concentration ratio) was not achieved for either 20 or 40 mg/kg levetiracetam, although there was a tendency towards equilibration. In contrast, for the 80 mg/kg group, equilibration was achieved by approximately 150 minutes. At equilibration the mean ± SEM CSF/serum levetiracetam concentration ratio was 1.09 ± 0.03 and mirrored that of the free fraction of levetiracetam.
<table>
<thead>
<tr>
<th>Rat No</th>
<th>Tmax (h)</th>
<th>Cmax (µmol/l)</th>
<th>AUC (µmol/l/hr)</th>
<th>t_{1/2} (h)</th>
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<td>96</td>
<td>603</td>
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<tr>
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<td>660 ± 49</td>
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<td>927</td>
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<tr>
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<td>889 ± 55</td>
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<td><strong>Mean ±sem</strong></td>
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<td>513 ± 30</td>
<td>3150 ± 211</td>
<td>4.5 ± 0.1</td>
</tr>
</tbody>
</table>

Table 5.2 Neuropharmacokinetic constants for CSF after intraperitoneal administration of levetiracetam (20, 40 and 80 mg/kg). Tmax = time to maximum concentration; Cmax = maximum concentration; AUC_{(0-8 hr)} = area under the concentration versus time curve; t_{1/2} = apparent elimination half-life.
Figure 5.5 Levitiracetam CSF:serum concentration ratio versus time profiles after 20 mg/kg ( ), 40 mg/kg ( ) and 80 mg/kg ( ) levitiracetam. Values are mean ± sem of 6 rats.
5.1.4 Discussion

Although as part of its preclinical development many studies were undertaken to determine the blood (plasma) pharmacokinetics of levetiracetam, there is a sparcity of published data. Furthermore, as far as we are aware, there are no published data describing the central neuropharmacokinetics of levetiracetam and this is the first report on the temporal interrelationship of levetiracetam serum pharmacokinetics and CSF neuropharmacokinetics in the rat.

The major findings of this study concern the temporal pharmacokinetics and neuropharmacokinetics of levetiracetam after acute administration (20, 40 and 80 mg/kg). After intraperitoneal administration, levetiracetam rapidly appeared in serum (mean Tmax, 0.25-0.50 hr) suggesting ready penetration from the peritoneal cavity. In addition, levetiracetam quickly appeared in CSF suggesting ready penetration of the blood brain-barrier. As might be expected Tmax values for the CSF compartment are somewhat larger (mean Tmax 1.33-1.92 hr) and represent the time of levetiracetam penetration from serum to brain and finally the CSF compartment. The dose dependent increase in serum and CSF levetiracetam concentrations, although not entirely linear in the CSF, after levetiracetam administration (20, 40 and 80 mg/kg) suggests that transport across the blood brain barrier is not rate limiting over the concentration range observed in the present study.

As was expected the efflux of levetiracetam from the CSF compartment was significantly slower (mean t½ range, 4.4-4.9 hr) than that suggested by serum concentrations (mean t½ range, 1.8-2.8 hr) and confirms similar observations with other antiepileptic drugs including carbamazepine, phenytoin, diazepam and milacemide (Lolin et al., 1994, Semba et al., 1993, Sokomba et al., 1988). This, in part, may be attributed to the continuing transport of levetiracetam from the blood compartment to the CSF compartment. The mean serum t½ range (1.8-2.8 hr) reported in this study is comparable to that recently reported for plasma (t½ range 2-3 hr) collected by orbital puncture of female Wistar rats administered levetiracetam intraperitoneally at a dose of 54mg/kg (Loscher et al., 1993).
The serum free/total serum levetiracetam concentration ratio (free fraction), as determined by ultracentrifugation, was 1.01±0.02 (mean ± sem) and was not time- or concentration-dependent over the concentration range of 92-502 μmol/l levetiracetam. As a drug free fraction of unity indicates that a drug is dissolved totally in plasma water and is not bound to blood proteins, it can be concluded that levetiracetam is not protein bound. That levetiracetam is not protein bound in serum is further emphasized by the observation that at equilibrium the CSF/serum concentration ratio (Figure 5.5, 80 mg/kg levetiracetam) is similar to that of the free fraction.

In conclusion the kinetics of levetiracetam in the rat is simple and thus dosing strategies in studies designed to elucidate its mechanism of action should be straightforward.
5.2 Levetiracetam chronic kinetic study

5.2.1 Experimental protocol

As in the chronic study carried out for CBZ the aim of the initial study was to establish the time taken for levetiracetam to reach steady-state concentrations in the blood and CSF. Male Sprague Dawley rats (n=6) were administered with 8 and 16 mg/kg/hr levetiracetam, respectively, via the osmotic minipump. Surgery involved implanting a blood catheter in the jugular vein, a CSF catheter in the cisterna magna and the osmotic minipump, containing levetiracetam, in the intraperitoneal cavity. The initial onset of the drug was delayed until approximately 48 hrs after surgery, by attaching a coiled tubing (PE 40), 97 cm in length, to the end of the minipump. CSF (30 µl) and blood (50 µl) samples were collected at 30 min intervals over an 8-10 hour period. Samples were stored at -70°C until required for analysis by HPLC, for levetiracetam content.

The previous study with levetiracetam dealt with single dose kinetics in serum and CSF compartments. The kinetics of repeat doses such as may be used in the treatment of epilepsy has not been previously established. Moreover, the simultaneous monitoring of CSF concentrations may give a more accurate indication of the concentrations at the receptor site (Greenblatt & Sethy, 1990). Therefore the aims of the second stage of this study were to determine whether repeat administration of levetiracetam may significantly alter serum and CSF kinetics which could lead to dangerous accumulation. This involved establishing levetiracetam concentrations in blood and CSF over seven days. For this study male Sprague Dawley rats were administered with 8 and 16 mg/kg/hr (n=6) levetiracetam, respectively, via an osmotic minipump. The surgical procedure involved implanting a blood catheter in the jugular vein, a CSF catheter in the cisterna magna and the osmotic minipump, containing levetiracetam, in the intraperitoneal cavity. On days 2, 4, 6, and 7 after surgery CSF (30 µl) and blood (50 µl) samples were simultaneously withdrawn every 30 min for a 6-8 hour period. Samples were stored at -70°C until required for analysis by HPLC for levetiracetam content.
5.2.2 Blood pharmacokinetics

Figures 5.6 and 5.7 show the mean serum concentration versus time profiles of levetiracetam, following levetiracetam administration (8 and 16 mg/kg/hr). Results are mean ± SEM for 6 rats. Levetiracetam was absorbed and first appeared in the blood compartment between 1.25-1.5 hr, after initiation of continuous intraperitoneal administration (Figure 5.6 and 5.7). Concentrations rose rapidly and essentially linearly with maximum concentrations achieved at approximately 7.5-9.0 (mean; 8.1±0.2) and 5.5-7.5 (mean; 6.5±0.2) hrs, for 8 and 16 mg/kg/hr post levetiracetam administration, respectively (Table 5.3). Mean Cmax values for levetiracetam were 284.8 ± 15.0 and 373.5 ± 10.7 for 8 and 16mg/kg/hr levetiracetam administration, respectively. Since both Tmax and Cmax values were determined from the graphs, and the first time point was at 30 minutes, these values may have been over and under estimated respectively. As levetiracetam was administered continuously during the period of sampling the Cpss max, Cpss min and Cpss ave concentrations were calculated, once steady-state concentrations were achieved (Table 5.4).

Steady-state concentrations for levetiracetam (8 and 16 mg/kg/h) were achieved at 9.0 and 7.5 hr respectively, post drug administration (Figure 5.6 and 5.7). At steady-state levetiracetam concentrations remained reasonably constant although some oscillations in concentrations is evident, for both dosing regimes (Figure 5.8 and 5.9).

5.2.3 CSF neuropharmacokinetics

Figures 5.6 and 5.7 in addition show the concurrent mean CSF concentration versus time profiles. Levetiracetam was not detectable in the CSF until the fourth time point, 90 minutes post-dose. Concentrations rose gradually and essentially linearly until steady-state was achieved and concentrations paralleled those observed in serum. Peak concentrations were achieved between approximately 7.5-9.0 (mean; 8.8±0.3) and 6.0-7.5 (mean; 7.1±0.3) hours post levetiracetam administration, 8 and 16 mg/kg/h, respectively (Table 5.3). The mean Cmax values for each dose were 217.3±11.6 and 328±17.8 μmol/l, respectively (Table 5.3).
Figure 5.6 Serum (---) and CSF (----) concentration versus time profiles of levetiracetam, post levetiracetam was administered at 8 mg/kg/hr. Values are means ± SEM of 6 rats.

Figure 5.7 Serum (---) and CSF (----) concentration versus time profiles of levetiracetam, post levetiracetam was administered at 16 mg/kg/hr. Values are means ± SEM of 6 rats.
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<th>Cmax (μmol/l)</th>
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<td><strong>284.8±15.0</strong></td>
<td><strong>8.8±0.3</strong></td>
<td><strong>217.3±11.6</strong></td>
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<td>7.5</td>
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<td><strong>328±17.8</strong></td>
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Table 5.3 Serum and CSF kinetic constants following intraperitoneal administration of levetiracetam
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<tr>
<td>Cpss ave</td>
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<td>26.3</td>
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</table>

Table 5.4 The mean maximum, minimum and average concentrations in the blood and CSF of levetiracetam (8 and 16 mg/kg/hr), during steady-state
Figure 5.8 Serum (○) and CSF (□) concentration versus time profiles of levetiracetam, post levetiracetam administration (8 mg/kg/hr). Values are mean ± SEM of 6 rats.
Figure 5.9 Serum (■) and CSF (□) concentration versus time profiles of levetiracetam, post levetiracetam administration (16 mg/kg/hr), over a seven day period. Values are mean ± SEM of 6 rats.
The concentration versus time profiles of levetiracetam in the CSF paralleled those of the blood compartment, and steady-state concentrations were achieved at approximately 9.0 and 7.5 hrs respectively, post drug administration. Once steady-state was achieved, Cpss max, Cpss min and Cpss ave concentrations in CSF were determined and were 187.7±27.6; 177.1±23.5; 182.4±15.1 and 298.7±15.5; 239.4±36.9; 269.1±22.3, following 8 and 16 mg/kg/hr, respectively (Table 5.4). Furthermore in the CSF compartment steady-state concentrations showed less variability than those in serum and were maintained over the remainder of the sampling period (Figure 5.8 and 5.9).

The CSF/serum concentration ratio reaches equilibrium status at the time point at which levetiracetam was first detected in both the blood and CSF compartments, for both doses (8 and 16 mg/kg/hr; Figure 5.10). The CSF/serum concentration ratio was similar to that observed in the acute study with levetiracetam. The CSF/serum concentration ratio on day one, for the 8 and 16 mg/kg dosages were 0.79 ± 0.03 (range; 0.6-0.98) and 0.72 ± 0.05 (range; 0.4-0.96), respectively.

5.2.4 Discussion

The previous investigation of levetiracetam pharmacokinetics dealt with single dose kinetics in the blood and CSF compartments. The present investigation deals with the temporal interrelationship of levetiracetam serum pharmacokinetics and central neuropharmacokinetics, post continuous infusion of levetiracetam via the osmotic minipump, in the rat. As in epilepsy, repeat chronic drug administration occurs, it is important to establish whether or not such administration will affect the kinetics of levetiracetam.

In contrast to single dose administration via the intraperitoneal cavity, levetiracetam administration (8 and 16 mg/kg/hr) via the osmotic minipump resulted in levetiracetam appearing in the serum compartment slowly, (mean Tmax 8.1±0.2 and 6.5±0.2, respectively). The difference in methodology used to administer levetiracetam can readily explain the difference since i.p. administration represents
a bolus administration whilst osmotic minipump administration represents a slow infusion administration. This is further emphasized by the fact that entry into the CSF compartment from the blood compartment were comparable for the two routes of levetiracetam administration.

Steady-state concentrations for levetiracetam were achieved during the first day, post drug administration (8 and 16 mg/kg/hrs; 9.0 and 7.5 hrs, respectively). These concentrations persisted for the remainder of the sampling period, although some oscillation in the concentrations were evident, with both dosing regimes. These data suggests little if any induction on hepatic microsomal activity, and are consistent with previous experiments which report no effect on the hepatic microsomal activity in rats by levetiracetam (Walker & Patsalos, 1995; Perucca & Bialer, 1996).

The dose-dependent increase in both serum and CSF levetiracetam, post levetiracetam administration (8 and 16 mg/kg/hr) suggests that transport across the blood brain barrier is not rate limiting over the concentration range observed in the present study. Furthermore, the levetiracetam CSF/serum concentration ratios suggest that equilibrium between the two compartments was rapid and was indeed evident at the time of first sampling. The rapid attainment of equilibrium between the brain and serum is in line with various pharmacokinetic characteristics of levetiracetam including (a) it is not protein bound, (b) it exhibits no specific binding to numerous peripheral tissues including heart, kidneys, spleen, pancreas, adrenals, lungs and liver (Noyer et al., 1995), and (c) its transport across the blood brain barrier is not rate limiting over the concentration range observed in the acute study and chronic studies.

Thus, following its continuous infusion levetiracetam shows simple and predictable kinetics. These data taken together with that of the acute study (previous investigation) would suggest that levetiracetam, in contrast to clinically established antiepileptic drugs (CBZ, phenytoin, valproic acid), exhibits ideal pharmacokinetics. Consequently, levetiracetam should be simpler to use clinically than these older currently available antiepileptic drugs. Eventually it is hoped that levetiracetam,
together with its ideal kinetics and potent antiepileptic properties will prove to be an important antiepileptic drug in the treatment of epilepsy in the not so distant future.
Chapter 6  Seizure Syndrome

6.1  Experimental protocol
The aims of this study were to characterise the tetanus toxin model of chronic limbic epilepsy in our laboratory and to further develop, characterise and validate the EEG monitoring of resultant seizures. Male Sprague Dawley (n=6) rats were injected with minute doses of tetanus toxin in the dorsal hippocampus. A bipolar electrode was then placed in the CA1 pyramidal cell layer of the right hippocampus. Initial EEG recordings were started 1-2 days after the injection of tetanus toxin and electrode implantation, and continued for a further 5-7 days. These data, served as confirmation of spontaneous epileptic seizures for each animal. Subsequently a minipump containing the drug or vehicle was implanted into the peritoneal cavity of the animal. Finally, continuous EEG and video recordings were taken for a further 7 days, and these data were used as control data for the comparison of the drug treated and control animals.

6.2  Behaviour during the seizure syndrome
Rats which were injected with tetanus toxin behaved as control animals until seizures started, whereupon, a general increase in irritability showed itself as difficulty with handling and a greater tendency to scratch the headstage when grooming.

Three types of epileptiform activity were recorded in vivo; inter-ictal spikes, polyspikes and seizures (Figure 6.1). Inter-ictal spikes and polyspikes, were not accompanied by any behavioural changes that could be identified. In contrast, seizures produced a variety of stereotyped behavioural changes which altered as the seizure syndrome progressed. The seizures have been divided into two types: non-generalised and generalised (Figure 6.2). The non-generalised category included behaviours such as immobility, vibrissal or eyelid twitching and gentle head nodding and effectively corresponded to a partial seizure. During such seizures the animals were generally not aware of their surroundings, as determined by their unresponsiveness to any external stimuli or handling. The generalised category
Figure 6.1 Three types of epileptiform activity recorded in vivo: a, b, c are examples of an inter-ictal spike, polyspike and a non-generalised seizure respectively. All traces were recorded from the CA1 region of the uninjected hippocampus. Note that the time scale for the traces varies.
Figure 6.2 Non-generalised (a) and generalised (b) seizures recorded in vivo. Both traces were recorded from the CA1 region of the uninjected hippocampus, of the same animal. The EEG trace of the non-generalised seizures was taken at the beginning of the seizure syndrome, during epileptogenesis, and trace b was taken at the end of the seizure syndrome.
included behaviour such as those described above but also rearing with or without forelimb clonus or falling. This was equivalent to a secondarily generalised seizure. Although the animals frequently fell backwards or sideways, they righted themselves immediately. The end of such seizures were usually signified by wet-dog shakes, purposeless chewing and a period of grooming. Some seizures developed into running or jumping seizures at which point the animals would race uncontrollably up and down their cage. Occasionally animals had seizures during which they walked with their bodies unusually low and elongated, and exhibited the usual facial symptoms of a non-generalised seizure. Recovery from this type of seizure usually occurred within minutes. After both types of seizures, the animals lay passively in their cages and seemed unaware of any external stimuli. Full recovery from both generalised and non-generalised seizures was normally indicated when the animals would resume normal activities, such as eating, grooming, walking around their cages, or dozing.

Typically before both types of seizures the animals had a characteristic blank expression and stood motionless with their ears held close to their heads. This phase lasted anytime between 10 to 20 seconds. Occasionally, however, animals awoke from sleep with a seizure. In this instance, the animals body would jerk to the motionless stance previously described.

6.3 Seizure course

Typically seizures tended to cluster with the first cluster starting between 1-2 days post operatively and lasting 2-3 days and the second starting between 3-5 days post-operatively and lasting 7-10 days. Peak seizure frequency occurred around 5-6 days post-operative i.e. during the second cluster (Figure 6.3). Seizure type evolved during each seizure cluster; non-generalised seizures occurred earlier and were later replaced by generalised seizures (Figure 6.3). Furthermore, the same pattern could be seen during the seizure syndrome with non-generalised seizures occurring at the beginning of the syndrome later to be replaced by generalised seizures. As the seizure type evolved the length of the seizures became gradually longer. At the beginning of the
Figure 6.3 Histograms representing the frequency of generalised (■) and non-generalised (□) seizures per 24 hours during the seizure syndrome. Each graph represents a control animal with no drug or vehicle administration. Note non-generalised seizures tended to occur at the beginning of the cluster and were followed by the development of generalised seizures.
seizure syndrome the length of non-generalised seizures was substantially shorter than that observed with generalised seizures (Figure 6.2). However, as the seizure syndrome progressed and non-generalised seizures were replaced by the generalised seizures, the difference in length became minimal. In 9 out 12 animals analysed, more seizures were observed during the night time than during the day period, but a Mann-Whitney rank sum test analysis of the data demonstrated that the difference was not statistically significant (p=0.908; Table 6.1). Day was defined as the period between 6 am and 6 pm.

6.4 Electroencephalographic activity

6.4.1 Control animal EEG

The EEG of control animals, injected with buffer, showed baseline low frequency field potential oscillations. The amplitude of the oscillations was dependent on the animal's activity; when asleep the baseline was relatively flat while movement around the recording cage resulted in large amplitude field potential oscillations in the 4-12 Hz range. These large amplitude field oscillations are commonly referred to as rhythmical slow wave activity (RSA) or theta. No epileptiform activity was recorded.

6.4.2 Epileptiform EEG activity

As mentioned previously, three different types of spontaneous epileptiform activity were recorded; inter-ictal spikes, polyspikes and seizures (Figure 6.2). Inter-ictal spikes lasted 25 to 100 ms and consisted of a high frequency oscillation. Polyspikes lasted 0.4-2 seconds and consisted of a number of inter-ictal spikes conjoined and superimposed on a slow negative field potential shift. The electrographic trace recorded during a seizure was composed of units which resembled inter-ictal or polyspikes with the difference that seizure activity continued for longer periods. Furthermore, the normal baseline hippocampal field potential oscillations seen when recording freely moving activity disappeared leaving a flat EEG trace between inter-ictal type events during a seizure.

The electrographic recording during a seizure was not constant. Instead there
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<td><strong>691</strong></td>
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Table 6.1 Mean number of seizures occurring during day and night time. The difference was not statistically different (p=0.908).
appeared to be an evolution of the hippocampal discharge. Therefore, the hippocampal discharge is divided for convenience into 5 stages. This classification, however, was not rigid; overlap between the different stages occurred and the time spent in each stage varied, from seizure to seizure and from animal to animal. Occasionally, some stages did not occur.

Examples of a generalised seizure in its various stages can be seen in Figures 6.4 to 6.9. As can be seen in figure 6.4 (a) the onset of the seizure is sudden. The hippocampal discharge during stage 1 is slightly irregular and rapid progression to stage 2 activity occurs (Figure 6.4 (b)). Stage 2 occurs less than one second after onset and consists of high frequency regular bursting (Figure 6.4 (b)). In stage 3 the high frequency electrographic activity seen in stage 2 becomes discontinuous and are replaced by bursts which resemble polyspikes (Figure 6.4 (c)). These polyspikes were normally separated by flat EEG traces (Figure 6.5). Stage 4 was where the seizure becomes generalised as was determined from the behaviour of the seizing animal using the video recording analysis. The discharges described in stage 3 lengthened or became continuous (Figure 6.6 (i)). During stage 4, the electrographic activity became irregular. In some seizures epileptiform activity stopped after stage 4 and the EEG would be quiescent for up to 30 seconds before small population EPSPs would appear and evolve into inter-ictal bursts. Alternatively, and in this instance, stage 5 inter-ictal type bursts evolved directly from stage 4 without the quiescent phase (Figure 6.6 (j)). Stage 5 could last up to 1.5 minutes (Figures 6.8 and 6.9). The end of the generalised seizure was usually signified by wet-dog shakes, purposeless chewing and a period of grooming (Figure 6.9). The large amplitude activity recorded at the end of the seizure (Figure 6.9 (k)) was due to movement artefact caused by the wet-dog shakes of the animal.

6.5 Discussion

The study of the epilepsies has been largely dependent upon the use of model systems, since for ethical considerations it is not possible to undertake for example intracellular recordings and microchemical analysis in the intact human brain.
Figure 6.4 Sudden onset of a generalised seizure (a) with progression to stage 2 less than one second after onset (b). 15 Hz activity can be seen during stage 2. Stage 3 begins at point (c) and consists of polypsikes (d) with flat EEG trace between bursts (e). These polysikes can last between 200-700 ms. All traces were recorded from the CA1 region of the uninjected hippocampus. Each trace is a continuation of the one above it.
Figure 6.5 Continuation of the same seizure illustrated in previous figure. The polyspikes lengthen (f) and the duration of the flat EEG between bursts reduces (g) as the seizure evolves from stage 3 to 4.
Figure 6.6  Continuation of the same seizure illustrated in the previous figures. Stage 4 begins at (h), during which the electrographic activity becomes irregular (i). Stage 5 begins at (j) and the electrographic activity becomes regular again. Stage 5 can last up to 1.5 minutes which can be seen in the following figures.
Figure 6.7 A continuation of stage 5 which began at point (j) in the previous figure.
Figure 6.8 Stage 5 continues.
Figure 6.9 Stage 5 ends here. The large amplitude activity at the end of the seizure (k) is due to movement artefact caused by clonic jerking of the body during seizure generalisation.
In the present chapter an epileptiform syndrome in rodents produced by injecting minute doses of tetanus toxin into the hippocampus, was characterised. The model is based on an adaption of a previously described epileptic animal model (Mellanby et al., 1977). The rat is exceptionally sensitive to injection of tetanus toxin in the region of the hippocampus, and injecting minute quantities of tetanus toxin leads to the development of an epileptiform syndrome (Mellanby et al., 1977). Despite the fact that tetanus toxin was first used over 30 years ago as a model of epilepsy, it is still utilised in some laboratories. It was first used to create chronic epileptiform events in 1962 by Carrea and Lanari who applied it to the cerebral cortex of dogs. Since then, tetanus toxin has successfully been applied to the hippocampus (rat, cat), substantia nigra (rat), thalamus (rat) and cerebral cortex, including motor cortex (dog and cat). However, the most frequent application of this model has been hippocampal injections in rat and to a lesser extent, in cat. The resulting complex partial seizure model probably results more from this injection site in limbic structures than from the properties of the toxin itself.

This particular epileptic model was chosen due to various reasons, namely; (i) the onset of seizures is rapid, i.e seizures may occur within a day after injection, (ii) subsequent to the injection, the seizures occur on a chronically recurrent basis over many months, allowing lengthy studies to be carried out, (iii) the model allows for continuous EEG and video monitoring of the animals and thereby enabling the investigator to determine seizure type and also to observe any abnormalities during the seizure syndrome, or any adverse side effects the animal may incur due to the administration of drugs. In many respects, the results of this study paralleled those of Mellanbys. However, due to differences in experimental protocol, it was necessary to further characterise this model and also to further develop, characterise and validate the EEG monitoring of resultant seizures. The alterations in experimental procedure, mentioned earlier included (i) the site of injection; unilateral as opposed to bilateral injection, dorsal versus ventral hippocampus, (ii) the absolute amount of toxin injected into the hippocampus of the animal, (iii) pre and post handling of the animals and finally (iv) the general husbandry conditions of the animals.
Probably the most important aspect when choosing a suitable animal model is how relevant your observations and answers are clinically. It has been previously reported by Mellanby et al., 1977, and also found during this thesis, that the nature of the seizures and the electroencephalographic changes observed during the seizure course with the tetanus toxin model, bare at least a superficial resemblance to those seen in human temporal lobe epilepsy. For instance, epileptic patients often experience an “aura”, which is usually reported as unpleasant, and frequently involves feelings of anxiety. This event experienced in man may be compared with the initial stage when the animals usually froze and had their ears laid back which can be considered to be an emotional response. There is also the stage in man which is characterised by oral automatisms and limb movements. This was also seen in the animals facial jaw movements, eg vibrissal or eyelid twitching and the purposeless chewing described at the beginning of both generalised and non-generalised seizures. Clonic movements of the forelimbs culminating in rearing and falling were also noted in the seizing animal during a generalised seizure. Another comparison which may be made is the postictal stage which, as in the corresponding stage in man, the animals appeared unresponsive to their surroundings or external stimuli. Moreover, the duration of each stage of the seizure was also similar to that seen in human temporal lobe epilepsy. Finally in some instances the animals woke suddenly with a seizure. This is a well known event in patients with temporal lobe epilepsy.

There are many controversial opinions among clinicians as to whether human temporal lobe epilepsy is associated with general behavioural disturbances. It is, therefore, interesting that the animals which were injected with tetanus toxin and had intermittent seizures, showed other behavioural disturbances in the intervals between seizures. During the course of the seizure syndrome the rats became increasingly irritated and in some cases vicious with minimal provocation. As the seizure course progressed and the number of seizures increased, the animals became less tolerant and resorted to biting if any attempt was made to handle or touch them. This would suggest that the abnormal behaviour was a consequence of the seizures rather than an effect of the toxin injected. Control animals which were similarly injected but with
saline showed no such adverse behaviours. This, however, was in direct contrast to the observations of Mellanby et al., (1977), who found that in most of the rats studied, hyperreactivity, hyperkinesis and aggressive behaviour were observed to occur several days before seizures were seen. The difference in experimental protocol, as mentioned earlier may have contributed to this difference.

The three types of epileptic activity recorded were interictal, polyspikes and a full blown seizure. Interictal and polyspikes normally occurred randomly between seizures and no abnormal behaviour was associated with these discharges. The EEG traces of non-generalised and generalised seizures differed in many respects: seizure length, number of stages involved and the length of the stages. It is also worth noting that generalised seizures also differed from other generalised seizures in the same aspects. Furthermore, it became increasingly difficult to distinguish non-generalised from generalised, using only the electrographic traces, as the seizure syndrome progressed. At this stage the video recordings analysis became more informative, as the seizure type was determined more on the basis of the behaviour of the animal during the seizure than on the EEG recording. However, in all experiments, the determination of seizure type was always confirmed using the video recordings, regardless of the stage of the seizure syndrome.

No statistical difference (p=0.908) was observed in the incidence of seizures recorded during the day and night time. Using a Mann-Whitney rank sum test, the difference in the median values among the two groups was not great enough to exclude the possibility that the difference was due to random sampling variability. Large variability between subjects was observed (range 65-201). This variability, however, is a well known characteristic of the tetanus toxin epilepsy model. As each animal was treated the same, very little could be done to reduce this between subject variability.
7.1 Carbamazepine in vivo study

7.1.1 Experimental protocol

This chapter deals with the determination of the antiepileptic properties of CBZ, continuously administered i.p., in the tetanus toxin model of epilepsy. The effects of CBZ and CBZ-E on seizure type, frequency and length, at concentrations measured in the blood and CSF samples during the CBZ chronic pharmacokinetic study (Chapter 4), were investigated. The methodology involved chronic administration of CBZ (4 mg/kg/hr), via the osmotic minipump, to epileptic rats (n=6), 5-7 days after the first seizure was witnessed. The surgical procedure involved injecting tetanus toxin into the dorsal hippocampus of the rat and allowing the animal to recover fully. Animals were then continuously observed using EEG and video recordings for 5-7 days, to confirm that epileptic seizures were occurring. Subsequently a second surgical procedure was undertaken for the implantation of the osmotic minipump. Continuous EEG and video recordings were taken again, for a further 7-8 days. The antiepileptic effect of CBZ and CBZ-E was determined by comparison of the drug group with the control group, during this period. A control animal was always investigated in parallel with a drug treated animal. The drug group was dosed with CBZ 4 mg/kg/hr, and the control group with the vehicle used to dissolve CBZ. It was decided not to investigate the CBZ and CBZ-E effects on seizures using the lower dose (2 mg/kg/hr), due to the very low concentrations of CBZ and CBZ-E detected, both in blood and CSF, during the CBZ chronic pharmacokinetic study (Chapter 4).

7.1.2 Antiepileptic effect of CBZ and CBZ-E

In these experiments the effect of continuous i.p. administration of CBZ via the osmotic minipump, to animals made epileptic with intrahippocampally injected tetanus toxin was studied. In figures 7.1 (a, b) and 7.2 (a, b) the history of the seizures experienced in six control and drug treated animals is illustrated, showing the median number of generalised and non-generalised seizures per day throughout the period that filming and EEG recordings were carried out. In addition the corresponding CBZ and CBZ-E concentrations, as determined from the chronic CBZ pharmacokinetic
Figure 7.1 The median number ± IQR (n=6) of generalised seizures (a) and non-generalised seizures (b) each day, in the control (■) and drug group (□□□) and the corresponding CBZ concentrations in serum (●) and CSF (□) of the drug group only. CBZ was administered at a rate of 4 mg/kg/hr.
Figure 7.2 The median number ± IQR (n=6) of generalised seizures (a) and non-generalised seizures (b) each day, in the control (■ ■ 1 ) and drug group ( □ □ ), and the corresponding CBZ-E concentrations in the serum ( - ▪ - ) and CSF ( - □ - ) of the drug group only.
study, in serum and CSF in the drug group, are illustrated. It is evident that CBZ and CBZ-E alleviated the number of generalised seizures during each day with the exception of day 5. A striking feature of these profiles is that this decrease is more pronounced when CBZ and CBZ-E concentrations are at their highest, i.e. before autoinduction occurred. In contrast, this was not apparent with the non-generalised seizures. On 4 of the 7 days more seizures were observed in the drug group than the control group, indicating that CBZ/CBZ-E had no effect on non-generalised seizures.

The antiepileptic effect of CBZ and CBZ-E on the total number of seizures following continuous infusion of CBZ is illustrated in figure 7.3. Although, there is a substantial reduction in the total number of seizures with the dosage used, this attenuation in seizure frequency did not reach statistical significance. The total mean number of generalised and non-generalised seizures, over this period, are reduced by 35.5 % and 12.6 %, respectively. Using a non-parametric two way analysis of variance CBZ and CBZ-E did not have a statistically significant effect on the seizure frequency of either generalised (p=0.617) nor non-generalised seizures (p=0.533). The difference is however, statistically significant (p=0.008) with generalised seizures, when the first couple of days of the week are considered. This is explained by the fact that autoinduction has not yet occurred and CBZ/CBZ-E concentrations are within the therapeutic range as defined for man. In contrast this is not observed with non-generalised seizures. Using the same analytical test and comparing drug treated and control groups, there was a insignificant reduction (p=0.566) in non-generalised seizure frequency. Comparison of generalised and non generalised seizure frequency before and after CBZ administration resulted in no significant difference (p=0.604; p=0.574 respectively). However, this comparison may not be appropriate as prior to CBZ administration the epileptic syndrome was in its developing stages whereas the period after CBZ administration the syndrome was fully established.

In addition the effect of CBZ and CBZ-E on the length of generalised and non-generalised seizures was also investigated. Control and drug group data were compared using a t-test. A Mann-Whitney rank sum test was carried out on any data not normally distributed. Table 7.1 represents the mean seizure length
<table>
<thead>
<tr>
<th></th>
<th>Control animals</th>
<th></th>
<th>Drug animals</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Generalised</td>
<td>Non-generalised</td>
<td>Generalised</td>
<td>Non-generalised</td>
</tr>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>Rat 1</td>
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<td>4.9</td>
<td>110.3</td>
<td>4.5</td>
</tr>
<tr>
<td>Rat 2</td>
<td>138.3</td>
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<td>55.0</td>
<td>14.5</td>
</tr>
<tr>
<td>Rat 3</td>
<td>95.4</td>
<td>3.7</td>
<td>86.0</td>
<td>1.2</td>
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<tr>
<td>Rat 4</td>
<td>103.0</td>
<td>4.1</td>
<td>73.3</td>
<td>21.9</td>
</tr>
<tr>
<td>Rat 5</td>
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<td>19.0</td>
<td>89.3</td>
<td>18.9</td>
</tr>
<tr>
<td>Rat 6</td>
<td>98.0</td>
<td>6.5</td>
<td>95.6</td>
<td>6.4</td>
</tr>
<tr>
<td>Overall mean</td>
<td>110.7</td>
<td>5.6</td>
<td>83.4</td>
<td>6.2</td>
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Table 7.1 Comparison of seizure length for generalised and non-generalised seizures in CBZ treated and control animals.
Figure 7.3 The cumulative effect of carbamazepine and carbamazepine 10, 11-epoxide, after carbamazepine administration (4mg/kg/hr) on the frequency of generalised and non generalised seizures, in the control without vehicle ( ), control with vehicle ( ) and drug treated ( ) groups, over a one week period. Values are median ± I.Q.R.
(seconds) of six rats from both the control and drug group. It is evident, from table 7.1 that a reduction in the generalised seizure length occurred (27.3 %). This difference in the mean values of the two groups was greater than would be expected by chance and was statistically significant (p=0.0001). Similarly a reduction was observed in the length of non-generalised seizures between the control and drug groups (7.9 %), however, it was considerably less than that observed with generalised seizure length. The difference in the median values among the two groups were not great enough to exclude the possibility that the difference was due to random sampling variability, and therefore this reduction proved to be statistically insignificant (p=0.414).

7.1.3 Discussion

The normal therapeutic range for CBZ and CBZ-E, as defined for man, is up to 50 µmol/l and 9 µmol/l, respectively. In the chronic pharmacokinetic study (Chapter 4), there was a significant reduction from the upper end of the therapeutic range followed by a gradual decreasing trend in serum and CSF CBZ and CBZ-E concentrations, once autoinduction had commenced. When peak concentrations for both CBZ and CBZ-E in serum and CSF were achieved the incident of generalised seizures, occurring per day, decreased dramatically (p=0.008). Also in some animals seizures were completely abolished (Figures 7.1 (a, b) and 7.2 (a, b)). As concentrations of CBZ and CBZ-E declined, due to autoinduction, both generalised and non-generalised seizures started to recur and were eventually as prominent as the control group. Thus a CBZ/CBZ-E concentration effect relationship was clearly evident with CBZ and CBZ-E exhibiting no effect on seizure frequency at the lower concentrations achieved. However, a greater antiepileptic effect was observed in the frequency or incidence of generalised seizures, per day, than that of non-generalised seizures. In fact the CBZ treated group had more non-generalised seizures than the control group on days 2, 3, 4 and 5. One possible explanation for this effect is that, CBZ and/or CBZ-E may be inhibiting propagation of seizure generalisation.

Although CBZ and CBZ-E did reduce the mean number of generalised and non-
generalised seizures during the week of recording (35.5% and 12.5% respectively), using a non-parametric two-way analysis of variance this difference proved to be insignificant (p=0.617 and p=0.533, respectively). This is in contrast to that previously reported by Hawkins et al., (1985) who found with their highest dose (60mg/kg, administered orally) there was a statistically significant reduction in the total number of overt seizures occurring throughout the syndrome. However, it is difficult to directly compare both investigations due to variations in the experimental conditions, namely, the mode of administration, time period studied and also dosing regimens used. (For a more detailed comparison of the CBZ study used in this thesis with that used by Hawkins et al., 1985 and of the possible clinical implications of the data see Appendix 1.) Also both serum and CSF CBZ and CBZ-E concentrations were determined in this investigation, whereas only serum CBZ concentrations were measured by Hawkins et al. (1985). In both man and rat CBZ-E has been shown to be equipotent to CBZ (Faigle et al., 1982). Therefore, in the present thesis the total effect of both CBZ and CBZ-E on seizure frequency, type and severity were investigated. Unfortunately, it was not feasible to increase the dose administered to the animal after autoinduction had commenced, due to a combination of the properties of CBZ and the minipump. That is the maximum amount of CBZ was dissolved already in the total volume allowed in the minipump.

The effect of CBZ and CBZ-E on seizure severity as determined by measurement of the seizure length was also investigated. CBZ and CBZ-E reduced the seizure length of both generalised and non-generalised seizures by 27.3 and 7.9%, respectively. These reductions were statistically significant for the generalised seizures (p=0.0001) but not for non-generalised seizures (p=0.414). Thus, although the antiepileptic effect of CBZ and CBZ-E in relation to the incidence or total number of seizures was not statistically significant, the animals experienced a general improvement in that their seizure severity was reduced. Daily handling and continuous monitoring of the animals showed that this “antiepileptic effect” was not accompanied by any obvious adverse side effects such as sedation.

Clinical studies of carbamazepine show, that whilst it is an effective antiepileptic
drug, it does not normalise the EEG. Although no detailed analysis of the EEG data were undertaken in this thesis, overall there was no apparent change in the frequency or incidence of interictal spiking. Thus, the epileptic tetanus toxin model appears to further reflect clinical epilepsy in relation to the action of CBZ.

In conclusion, CBZ administered via the osmotic minipump, exhibited antiepileptic properties in the tetanus toxin-induced hippocampal epilepsy, namely (i) it attenuated the incidence of generalised seizures occurring per day while peak concentrations of CBZ and CBZ-E were achieved and (ii) it reduced the overall length of generalised seizures, although this reduction did not achieve statistical significance in relation to total number of generalised seizures and (iii) it reduced seizure length of non-generalised seizures but again this effect was not statistically significant.
8.1 Levetiracetam in vivo study

8.1.1 Experimental protocol

The aims of the in vivo levetiracetam study was to investigate if the putative AED, levetiracetam exerted antiepileptic properties in the tetanus toxin-induced epilepsy model. Levetiracetam's effects on the seizure type, frequency and severity, at concentrations measured in serum and CSF during the levetiracetam chronic pharmacokinetic study (Chapter 5), were determined. The methodology involved chronic administration of levetiracetam (8 and 16 mg/kg), via the osmotic minipump, to epileptic rats (n=6) 5-7 days after the first seizure was witnessed. The surgical procedure involved injecting tetanus toxin into the rats hippocampus and allowing the animal to fully recover. After which, the animal was continuously observed using EEG and video recordings for 5-7 days, to confirm that seizures were occurring. Subsequently, a second surgical procedure was then undertaken for the implantation of the osmotic minipump, containing either levetiracetam or vehicle. Finally, continuous EEG and video recordings were recorded for a further 7-8 days.

8.1.2 Antiepileptic effect of levetiracetam

Figures 8.1 (a, b) and 8.2 (a, b) illustrate the history of the seizures experienced in six control and drug treated animals, showing the mean number of generalised and non-generalised seizures per day throughout the EEG and video recording period. In addition the corresponding levetiracetam concentrations, as determined from the chronic levetiracetam kinetic study, in serum and CSF of the drug treated group, can be seen. It is evident from these data that levetiracetam suppressed seizures at both doses of levetiracetam. In the case of the lower dosage, the mean number of generalised seizures per day was reduced with the exception of day 4. The extent of seizure reduction varied from day to day, with the lowest and highest antiepileptic effect detected during days 5 and 7, respectively. The mean number of non-generalised seizures were also reduced in five of the seven days. However, seizure suppression was not as substantial with the non-generalised seizures as it was for the generalised seizures. A similar pattern of seizure suppression was observed
Figure 8.1 The median number ± IQR (n=6) of generalised seizures (a) and non-generalised seizures (b) each day, in the control (■■■) and drug group (□□□) and the corresponding levetiracetam concentrations in serum (○○○) and CSF (□□□) in the drug group only. Levetiracetam was administered at a rate of 8 mg/kg/hr.
Figure 8.2. The median number ± IQR (n=6) of generalised seizures (a) and non-generalised seizures (b) each day, in the control (■) and drug group (□) and the corresponding levetiracetam concentrations in serum (●) and CSF (□) of the drug group only. Levetiracetam was administered at a rate of 16 mg/kg/hr.
for the higher dose of levetiracetam, and thus the antiepileptic effect of levetiracetam was dose-dependent.

Figures 8.3 and 8.4 summarises the antiepileptic properties exerted by levetiracetam over the seven days analysed. While there is an apparent reduction in the mean total number of seizures with both dose regimens, this did not reach statistical significance, with the lowest dose. Following levetiracetam administration (8 mg/kg/h) the generalised and non-generalised seizures were reduced by 39.1 % and 35.8 %, respectively. However, using a non-parametric two way analysis of variance these reductions were statistically insignificant, p=0.071 and p=0.635, respectively. Following levetiracetam administration (16 mg/kg/h) the generalised and non-generalised seizures were reduced by 56.7 % and 41.2 %, respectively (Figure 8.4). Using the same statistical analysis as previously mentioned the difference was statistically significant with generalised seizures (p=0.0004) and statistically insignificant with non-generalised seizures (p=0.525).

The antiepileptic effect of levetiracetam (8 and 16 mg/kg/h) on the length of both generalised and non-generalised seizures was also determined (Tables 8.1 and 8.2). Control and drug group data were analysed and compared using a t-test analysis. A Man-Whitney rank sum test was carried out on any data not normally distributed. A 35.7 % and 19.9 % decrease in the length of generalised seizures was observed, between the control and drug treated group, after 8 and 16 mg/kg, respectively. The difference in the median values of the two groups was greater than would be expected by chance and therefore there was a statistical significant difference with both doses (p=0.0001 and p=0.0001, respectively). However, no difference in seizure length was detected with non-generalised seizures with either dose. That is to say the difference in the median values among the two groups, was not great enough to exclude the possibility that the difference was due to random sampling variability.
## Seizure length (Control animals) vs. Seizure length (Levetiracetam treated animals)

<table>
<thead>
<tr>
<th></th>
<th>Seizure length (Control animals)</th>
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<th>Seizure length (Levetiracetam treated animals)</th>
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<tbody>
<tr>
<td></td>
<td>Generalised</td>
<td>Non-generalised</td>
<td>Generalised</td>
</tr>
<tr>
<td></td>
<td>Mean ±SEM</td>
<td>Mean ±SEM</td>
<td>Mean ±SEM</td>
</tr>
<tr>
<td>Rat 1</td>
<td>119.3 ± 4.9</td>
<td>20.6 ± 1.2</td>
<td>63.6 ± 1.4</td>
</tr>
<tr>
<td>Rat 2</td>
<td>126.6 ± 9.7</td>
<td>22.9 ± 2.1</td>
<td>75.6 ± 3.8</td>
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<tr>
<td>Rat 3</td>
<td>85.8 ± 12.3</td>
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<td>Rat 4</td>
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<tr>
<td>Rat 5</td>
<td>87.8 ± 1.9</td>
<td>39.6 ± 5.5</td>
<td>82.9 ± 8.0</td>
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<tr>
<td>Rat 6</td>
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<td>32.1 ± 3.3</td>
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<tr>
<td>Mean</td>
<td><strong>102.1 ± 3.9</strong></td>
<td><strong>25.1 ± 1.1</strong></td>
<td><strong>65.7 ± 2.3</strong></td>
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</tbody>
</table>

Table 8.1 Comparison of seizure length of generalised and non-generalised seizures in levetiracetam (8 mg/kg/hr) treated and control animals.
<table>
<thead>
<tr>
<th></th>
<th>Seizure length (Control animals)</th>
<th>Seizure length (Levetiracetam treated animals)</th>
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<tbody>
<tr>
<td></td>
<td>Generalised</td>
<td>Non-generalised</td>
</tr>
<tr>
<td></td>
<td>Mean ±SEM</td>
<td>Mean ±SEM</td>
</tr>
<tr>
<td>Rat 1</td>
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</tr>
<tr>
<td>Mean</td>
<td>130.8 ± 3.0</td>
<td>105.1 ± 4.9</td>
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</tbody>
</table>

Table 8.2 Comparison of seizure length of generalised and non-generalised seizures in levetiracetam (16 mg/kg/hr) treated and control animals.
Figure 8.3 The effect of levetiracetam, after levetiracetam was administered (8mg/kg/hr), on the total incidence of generalised and non-generalised seizures in the control without vehicle ( ), control with vehicle ( ) and drug group ( ) over a one week period.
Figure 8.4 The effect of levetiracetam, after levetiracetam was administered (16mg/kg/hr), on the total incidence of generalised and non-generalised seizures in the control without vehicle, control with vehicle and drug group over a one week period.
Levetiracetam (8 and 16 mgkg$^{-1}$ hr$^{-1}$) administration to the in vivo tetanus toxin epilepsy model, led to attenuation in the maximum number of spontaneous seizures occurring per day, and in the total number of seizures occurring over the total period investigated. Although this anticonvulsant effect was dose dependent, the only statistically significant effect was the reduction in seizure incidence following levetiracetam administration at the higher dose. Furthermore, levetiracetam had a greater effect on generalised seizure duration than incidence. At both doses a significant reduction in overall seizure length was seen. However, no significant effect on the duration of non-generalised seizures was observed. That is to say the difference in the median values among the two groups, was not great enough to exclude the possibility that the difference was due to random sampling variability. Additionally, levetiracetam did not cause any alteration in the seizure syndrome pattern, i.e. non-generalised seizures were more dominant at the beginning of the syndrome. These data suggest that levetiracetam appears to prevent or severely inhibit seizure generalisation in the tetanus toxin model.

There is also evidence of an apparent loss of effect during the chronic administration of levetiracetam, on days 4 and 5 in our study. This may be explained, at least in part by the development of tolerance by the animals to the antiepileptic effect of the drug. Determination of levetiracetam in blood and CSF indicated that this tolerance was not due to declining drug concentrations. However, further work is needed to determine the exact cause of this reduced effect following chronic administration of levetiracetam.

Although the two dose experiments were not originally intended to constitute a dose-dependent response curve: the strategy was to try to find a dose with a reliable anticonvulsant effect, a tendency towards a dose dependent response was observed. Treatment with the higher dosage resulted in a greater attenuation in the incidence in the total number of generalised and non-generalised seizures.

The kinetic profiles of levetiracetam correlate well with the findings of the anticonvulsant study. The major findings of the pharmacokinetic study demonstrate that steady state
levetiracetam concentrations in serum and CSF were achieved by the second day of sampling and these concentrations remained for the remainder of the sampling period. The serum concentrations of levetiracetam which we found in our rats were also comparable to those found to be effective in the clinical situation. An essential requisite of any novel anticonvulsant drug proposed for further development is that there should be a wide separation between effective anticonvulsant doses and those causing neurotoxicity, such as sedation, muscle relaxation and motor incoordination. Although not systematically investigated levetiracetam appears to fulfill this requisite. Daily handling and observation of the animals showed that any antiepileptic effect observed following levetiracetam administration occurred without obvious adverse side effects.

Tetanus toxin acts as a potent clostridial neurotoxin, blocking preferentially the presynaptic release of GABA and glycine. Previous studies (Whittington & Jefferys, 1994; Jordan et al., 1991; Empson et al., 1993) have reported a decreased release of GABA and in the size of IPSPs, which both stem from the cleavage of synaptobrevin by tetanus toxin. However, as the epileptogenic effect of tetanus toxin far out lasts the impairment of GABA release, a more complex action of tetanus toxin than just the blockade of inhibitory transmission must exist (Whittington & Jefferys, 1994). Although inhibition in the ipsilateral focus drops to 10% of controls during the first 2 weeks, the loss of inhibition in the contralateral mirror focus never drops below 50% (Empson et al., 1993; Whittington & Jefferys, 1994). This may suggest that the inhibitory neurons are not recruited by the hippocampal network, either because they are less excitable or because the synapses that excite them are impaired (Bekenstein et al., 1993, Sloviter, 1991; Whittington & Jefferys, 1994). Previous studies with levetiracetam have explored the possibility of whether levetiracetam exerts its anticonvulsant effects via an interaction with GABAergic mechanisms (Margineanu & Wulfert, 1995; Loscher et al., 1996). It was concluded from these investigations that although some interaction could exist between the levetiracetam binding site and the \( \text{GABA}_{\alpha} \) receptor complex, these interactions are possibly indirect. Such conclusions were reached because levetiracetam was found to be inactive at \( \text{GABA}_{\alpha} \) binding sites in radioligand studies (Loscher et al., 1996), and in pharmacological studies levetiracetam did not interact directly with the
GABAA/benzodiazepine receptor chloride ionophore complex (Margineanu & Wulfert, 1997). Our data somewhat confirm these results as levetiracetams anticonvulsant effect in the tetanus toxin model is unlikely to be a direct effect on GABA as this would have expected to decrease the incidence of seizures much more.

Other studies by Loscher et al., 1996, investigated the effects of various levetiracetam doses on GABA levels and the activities of GABA synthesizing and degrading enzymes. This study involved determining the GABA turnover in brain regions at different time points following levetiracetam administration and demonstrated levetiracetam induced alterations in the GABA system in several rat brain regions. As both increases and decreases in the GABA synthesizing enzyme glutamic decarboxylase (GAD) and the GABA degrading enzyme GABA transaminase (GABA-T) were found it is not likely that these alterations are a direct effect of levetiracetam but rather a consequence of post-synaptic changes in either GABAergic or other neurotransmitter-related systems.

Finally previous reports on levetiracetams highly stereoselective antiepileptic activity led to the investigation of a possible presence of a specific binding site for levetiracetam in the rat CNS by Noyer et al., 1995. These studies revealed that levetiracetam labels a single class of binding sites with modest affinity and with a high capacity in several brain regions. The binding of levetiracetam is also confined to the synaptic plasma membranes in brain regions such as the hippocampus, cortex, cerebellum and striatum, as no specific binding was observed in a range of peripheral tissues including heart, kidneys, spleen, pancreas, adrenals, lungs and liver. Levetiracetam bound to hippocampal membranes with modest affinity ($K_d = 780 \pm 115 \text{ nM}$) and high binding capacity ($B_{max} = 9.1 \pm 1.2 \text{ pmol/mg protein}$). Similar $K_d$ and $B_{max}$ values were obtained with the cortex, cerebellum and striatum. However, the affinity of levetiracetam for these sites was about 1000 times less than the concentrations achieved in the CSF compartment in our pharmacokinetic study. Together these results therefore suggest that levetiracetams binding site does not play a sole role in the anticonvulsant activity of the drug.
8.2 Levetiracetam in vitro study

8.2.1 Experimental protocol

The aim of the present in vitro study was to further elucidate the mechanism of action of levetiracetam. Levetiracetam was tested for its impact on seizure-like events and interictal bursting by determining the extent levetiracetam reduced (i) the length of the evoked epileptic discharges and (ii) the number of interictal bursts observed between these evoked seizure-like events. Epileptic discharges were evoked in slices from the hippocampus of adult rats, when inhibition was blocked acutely via the bath application of bicuculline methiodide (BMI 15 μM) and potassium ([K⁺]₀) raised to 5 mM.

Male Sprague Dawley rats (n=6) were anaesthetised via i.p. injection of hypnorm and hypnovel mixture, and killed by cervical dislocation. The skull was opened and the brain rapidly removed and glued upside down for the preparation of horizontal slices of the ventral hippocampus. The glued brains were immersed in ice-cold, oxygenated ACSF and slices cut, 450 μm thick. Slices were placed in an interface recording chamber and maintained at 30-35 °C. Four evoked responses were recorded consecutively as control data before the application of levetiracetam (200 and 400 μmol/l respectively). Slices were left for a further 30-45 minutes to acclimatise, before recording was recommenced. Levetiracetam was rinsed out, using ACSF, and the slices were again left for 1-1.5 hr before recording was resumed. The levetiracetam concentrations used in this study were based on concentrations observed in the CSF, during the chronic pharmacokinetic study described earlier.

8.2.2 Seizure-like events recorded in ventral slices

Extracellular recordings in CA1 from slices bathed in BMI and 5 mM KCL showed long lasting discharges with a distinct 2-phasic firing pattern. These two phases were ascribed primary and secondary (Figure 8.5). Typically an entire discharge usually lasted 15-30 seconds, but could last as long as 90 seconds, mainly as an ongoing rhythmic firing secondary burst. Most of the seizure-like events lasted approximately 20 seconds. Short seizure-like events differed from longer ones only in the length of the train of secondary bursts. Primary burst and the individual secondary bursts did not show any differences.
Figure 8.5 Epileptiform activity recorded from the CA1 pyramidal cell layer of the ventral hippocampus, showing long lasting discharges (15-30 seconds) (a) and (b), with a distinct 2-phasic firing pattern (c)
Figure 8.6 Extracellular traces were elicited with a single pulse stimulation delivered to the perforant pathway, and recorded from the CA1 of ventral hippocampus.
Primary bursts presented always as a unique polyspike event lasting 90-400 ms. The primary burst was immediately followed by an ictal-form event which slowly decayed. The secondary burst started as a train of rhythmic discharges. Each secondary burst lasted 50-75 ms and occurred at a peak frequency of 2-8 Hz. Thus 100-350 ms of non-activity typically occurred between the two bursts of activity. The train of secondary bursts started with its peak frequency or gained it within the first second, after which it gradually slowed down until the firing brake off was normally at a frequency of 0.5-3 Hz depending on the peak frequency. The faster firing seizure-like events stopped at higher frequencies.

All of the seizure-like events measured from the CA1, were elicited with single pulse stimulation, delivered to the perforant pathway (0.2 ms, 10-100 V; Figure 8.6). The described firing pattern of seizure-like events was not unique to evoked responses. Quite frequently touching the slice with the stimulating electrode in the process of its placement in the slice elicited a similar discharge without any electrical pulse. Fully spontaneous occurring seizure-like events were also recorded, but these were a rare event. Spontaneous seizure-like events resembled in all the described characteristics, i.e distinct phases and duration. Additionally to the described seizure-like events, disinhibition induced in all slices spontaneous firing of interictal bursts at a rate of 1-40 per minute. These interictal bursts which lasted 50-500 ms overall, very much resembled secondary bursts in the described seizure-like events.

Using 5 mM KCl and 10-20 μM BMI seizure-like events could be recorded in 36 out of 54 (66 %) slices from the ventral hippocampus. These seizure-like positive slices came from 7 out of 9 animals used (78 %), indicating that this *in vitro* model as reasonably reliable for the induction of seizure-like events. However, in some experiments the seizure-like events could only be evoked in some of the slices from the same rat. Hence the lower success rate for the slices compared to that for the experiments (rats used).

### 8.2.3 Levetiracetams effect on seizure-like events

Figure 8.7 illustrates the antiepileptic effect of levetiracetam on the seizure-like events recorded from the CA1 of the ventral hippocampus. Both concentrations of levetiracetam
Figure 8.7 Levetiracetams (200 and 400 μmol/l) effect on the length of seizure-like events recorded from the CA1 of the ventral hippocampal slice.
(200 µmol/l and 400 µmol/l) reduced the overall length of the seizure-like events but did not effect the distinctive 2-phasic firing pattern. The data from the control and drug treated slices were analysed and compared using a t-test, to determine if this reduction was statistically significant. A Man-Whitney rank sum test was carried out on any data that failed the normality test. The length of the seizure-like events was reduced by 33 % and 47 %, following accumulative application of 200 and 400 µmol/l concentrations. The difference in the median values of both groups was greater than would be expected by chance and therefore there was a statistically significant reduction in seizure length at both concentrations (p= <0.0001 and p= <0.0001, respectively). The length of the seizure-like events during the rinse out period was also compared to that of the control group. Using the same statistical criteria the difference in the median values among the two groups were not great enough to exclude the possibility that the difference was due to random sampling variability. Therefore there was not a statistically significant difference in seizure length between the control and the rinse out data. This indicates that levetiracetam must have been completely washed out of the slices and that the difference observed earlier was in fact due to the presence of levetiracetam.

Figure 8.9 demonstrates similarities of epileptic activity recorded in vivo and in vitro generated via injection of minute dose of tetanus toxin in the rat hippocampus and bath application of bicuculline and raised ([K+]o) to hippocampal slices. Both interictal spikes and polyspikes recorded in vivo and in vitro showed many similarities. In addition, the onset of both in vivo and in vitro epileptic discharges was sudden. With both spontaneous and evoked epileptiform events, a prolonged inter-ictal type burst was recorded followed by irregular discharges and rapid progression to stage 11 activity (Figure 8.9 (c)). Stage IV and V of the seizures recorded in vivo were mainly associated with seizure generalisation. The electrographic activity recorded during stage 5 showed a long phase of rhythmic bursts which could last variably from several seconds up to 2 minutes. These recordings were similar to the secondary train of rhythmic discharges recorded in vitro (Figure 8.9 (d)). The frequency of these recordings were however significantly different. The in vitro discharges occurred at a much slower frequency than the equivalent in vivo recordings.
Figure 8.8 Effect of levetiracetam (200 and 400 μmol/l) on the mean number of interictal bursts recorded between evoked seizure-like events. Values are mean±SEM from 6 slices.
Figure 8.9 Comparison of *in vivo* and *in vitro* recordings at various stages of an epileptic seizure: (a), (b), (c) and (d) represent an interictal spike, a polyspike, the sudden onset and the latter stage of a seizure, respectively, recorded from an intact brain (*in vivo*) and a hippocampal slice (*in vitro*).
8.2.4 Discussion

Levetiracetam exhibited profound anticonvulsant properties in the acute disinhibition model. Bicuculline is a commonly used convulsant, generally thought to cause seizures via reduction of inhibitory neurotransmission through blockade of \( \gamma \)-aminobutyric acid (GABA\( _{\text{A}} \)) receptors, although other mechanism are involved. Decreased inhibition of neuronal pathways causes an increase in the number of neurones firing simultaneously and hence hypersynchronous discharges of a large population of neurones results, which is similar to that which can be observed in human epilepsy. With regard to our experiments bath application of bicuculline (15 \( \mu \text{M} \)) and elevated potassium (KCl; 5 mM) to hippocampal slices produces trains of population bursts which lasted several seconds.

Many features, such as the fast AMPA receptor mediated, excitatory transmitter system, the recurrent excitatory collaterals and the intrinsic neuronal properties, that enable the disinhibited hippocampal slice to generate brief interictal bursts, will also be involved in generating the seizure-like events. However, the time scale of these components cannot explain the duration of the seizure-like events and the synthesis of the secondary bursts into a long train of after discharges. Other factors must be additionally involved, and must become activated before the initiation of the secondary bursts and persist during the entire seizure-like event. Two factors fulfilling these requirements that have been observed in seizure-like events is the rise in extracellular potassium (Dr. Cornelius Borck, personal communication) and the increase of spontaneous synaptic activity. Together with the other features of a disinhibited slice, high potassium concentrations and increased levels of synaptic activity may enable the CA1 network to fire these long lasting epileptic discharges. Both aspects are closely linked together; the strong activation of CA1 during primary and secondary bursts causes changes in \( [\text{K}^+]_0 \) and one of the effects of elevated potassium is the increase in spontaneous synaptic activity (Dr. Cornelius Borck, personal communication).

Previous studies carried out by Birnstiel et al., 1997 have examined the effect of levetiracetam on bicuculline induced epileptiform bursting and on epileptiform activity...
generated by the glutamate receptor agonist N-Methyl-D-Aspartate (NMDA). Levetiracetam had no effect on the size of the burst induced by NMDA but did decrease bursting frequency. Furthermore, preincubation with levetiracetam severely inhibited the development of epileptiform bursting in response to synaptic stimulation in the presence of bicuculline. However, once bicuculline induced bursting was established, levetiracetam was considerably less effective. The epileptiform bursts induced after preincubation with levetiracetam hardly exceeded the size of a normal synaptic potential, whereas, the number of action potentials and the area under previously established bursts were significantly reduced by levetiracetam, but not a reduction in burst duration (Birmstiel et al., 1997). Birmstiel explains the prevention of the epileptiform bursting by levetiracetam, by levetiracets act to prevent the formation of recurrent excitatory synaptic connections, by an unknown mechanism. The results here demonstrate that under our experimental conditions, levetiracetams antiepileptic effect, involved attenuation of the duration of firing of secondary bursts during the seizure-like events but not the distinctive bi-phasic firing pattern, suggesting that one of the possible mechanisms of levetiracetam is it may inhibit neuronal hyperexcitability via inhibition of the process involved in driving the long train of secondary bursts. As mentioned earlier, potassium may play a crucial role in the transition from interictal activity to seizure-like events. However, the potassium hypothesis certainly does not explain all the different phenomena of seizures (many more mechanisms can be expected to be involved). Therefore, further in vitro studies are needed to characterise the exact process underlying these secondary bursts and to determine how levetiracetam affects these mechanisms.

Finally, it is important to note that levetiracetam may be exhibiting anticonvulsant properties via different processes depending on the mechanism used to produce the epileptiform activity. Mechanisms other than those described for the hippocampal slice will be involved in the living animal. The different brain structures, which eventually become involved in a seizure, will modulate the firing pattern and thereby the recorded traces. For instance the difference in the frequency of the in vivo and in vitro recordings is probably due to the presence of many more neuron connections left intact in the whole
animal. However, with respect to the results of this study several similarities in the epileptiform events recorded during the *in vivo* and *in vitro* investigations were observed. For instance, the initial onset of the epileptiform activity recorded *in vivo*, when the animal experienced a generalised seizure was homogenous to the seizure-like events recorded *in vitro*. Additionally, the electographic activity recorded *in vivo*, from the dorsal hippocampus, during stage 5 of a generalised seizure showed a long phase of rhythmic bursts at the end of the seizure, which was equivalent to those of the secondary bursts recorded *in vitro* from the ventral hippocampus slice. Electrographically, the generalised seizures recorded *in vivo* showed, after a phase of building up and seizure initiation, a distinction between a 'tonic' phase with sustained, high frequency neuronal firing, and a 'clonic' phase, similar to the train of secondary bursts. This clonic phase could last several minutes and was sometimes, but not always associated with clonic movement. Unfortunately with the implanted electrodes, field potentials were recorded only from the hippocampus, and no direct evidence could be gained therefore about the spread of epileptic activity. However, the occurrence of these rhythmic bursts without behavioural signs suggest a rather limited epileptic activity, concentrated around the focus induced by the intrahippocampal tetanus toxin injection. This is a hint that rhythmic bursts, the equivalent of secondary bursts *in vitro*, may be generated locally *in vivo*. Thus, the hippocampal slice preparation not only preserves the basic physiology of the hippocampus, but also mirrors epileptic conditions realistically, when compared to *in vivo* models. Therefore, the morphological similarities between the secondary bursts recorded *in vitro* and the rhythmic bursts recorded *in vivo* make similar underlying mechanisms rather likely. The fact that levetiracetam exhibits anticonvulsant properties in both the *in vivo* and *in vitro* models in a similar fashion supports this possibility. Levetiracetam decreased the severity of the generalised seizures in the tetanus toxin model by reducing their over all length. This reduction was not seen with non-generalised seizures. In addition stage 5 epileptiform activity was only recorded during seizure generalised.

In summary the main findings during this investigation concern the antiepileptic properties exhibited by levetiracetam in two disinhibition models of limbic epilepsy. The
key observations were, treatment with levetiracetam resulted in (i) a substantial attenuation in the duration of epileptic activity recorded both \textit{in vivo} and \textit{in vitro}, and (ii) a significant reduction in the incidence or frequency of generalised seizures recorded \textit{in vivo}, while exhibiting no antiepileptic activity on non-generalised seizures. Thus, levetiracetam exhibits profound inhibitory effects upon hippocampal neural activity at both the cellular (\textit{in vitro}) and circuit (\textit{in vivo}) levels, and attenuates epileptiform bursting induced in the tetanus toxin model and the hippocampal slice model. This data suggests that levetiracetam may act by severely inhibiting seizure generalisation. In conclusion, the data presented in this report convincingly demonstrate levetiracetams potent anticonvulsant properties, low neurotoxic side effects and simple pharmacokinetics. Together with such features we believe that levetiracetam may play a crucial role in future treatment of epilepsy.
9.1 Search for new AEDs

Epilepsy is one of the most common neurological disorders, with a prevalence of 5 cases per 1000 of the population (Hopkins, 1987). Existing AEDs, as monotherapy regimens, can render approximately 80% of newly diagnosed patients seizure free (Shorvon & Reynolds, 1982). However, for the remaining 20% of patients, currently available treatment is unsatisfactory either because seizures are not adequately controlled or because side effects are intolerable. Additionally, these patients are usually prescribed polytherapy regimens and this can be associated with problematic pharmacokinetic interactions (Patsalos & Duncan, 1993). Other problems with traditional AEDs include a narrow therapeutic ratio, development of tolerance and enzyme induction. There is, therefore, a growing need for new AEDs with improved risk/benefit ratios.

The search for new AEDs is a major area in its own right. In its early stages it depends largely on screening of candidate drugs on a limited number of animal models. However, the last two decades have seen an enormous and fascinating increase in AEDs development. The current standard AEDs, such as barbiturates, phenytoin, carbamazepine and ethosuximide were discovered, or stumbled upon in some instances, in the first half of this century and rapidly introduced into clinic. Thereafter, very little happened, until basic research in epilepsy brought a new wave of rational drug design (Rogawski et al., 1990). This basic research in epilepsy included the testing of compounds in animal seizure models, and resulted in a greater understanding of the molecular basis of seizures and, more importantly, a greater understanding of clinical pharmacokinetics. The first phase of pharmaceutical research focused on the inhibition aspect of epilepsy and resulted in the development of various chemicals with different mechanisms of action to enhance GABA-ergic transmission (Porter et al., 1992). Then the newly discovered properties of the NMDA receptor shifted much of the attention towards a reduction of excitatory, glutamatergic transmission (Rogawski, 1992). In contrast to these new approaches, most of the standard drugs act via effects on intrinsic neuronal properties and
modulate membrane conductances, involving mainly Na⁺ currents. This still remains a viable strategy for developing new AEDs (Upton, 1994).

9.2 Pharmacokinetic properties of new and old AEDs
An understanding of clinical pharmacokinetics has lead to (i) the improved clinical use of established AEDs, (ii) modifications of established AEDs to create drugs with better pharmacological profiles, and (iii) the early characterisation of the clinical pharmacokinetic profiles of new AEDs. Desirable pharmacokinetic properties for an AED include high oral bioavailability, negligible plasma protein binding, efficient penetration across the blood-brain barrier, a half-life compatible with once or twice daily dosing, and linear kinetics. Lack of biotransformation is also advantageous as it is normally associated with predictable kinetics and low susceptibility to drug interactions (Perucca & Pisani, 1991). Unfortunately no such drug exists, but recently several new AEDs, namely vigabatrin, lamotrigine, gabapentin, oxcarbazepine, topirimate, felbamate, zonisamide and tiagabine, have been marketed.

Compared with older agents, many of the new drugs exhibit simple pharmacokinetics. This is especially true for vigabatrin and gabapentin, which are renally eliminated and have a low interactive potential (Haegele & Schechter, 1986; Rimmer & Richens, 1989; Bartoszyk et al., 1988; Schmidt, 1989). In fact, several of the older drugs are far from ideal in their pharmacokinetic properties. For instance, phenytoin, in particular, shows variable bioavailability, extensive plasma protein and saturable metabolism (Richens, 1979), while valproic acid shows non-linear kinetics, due to concentration-dependent plasma protein binding as well as a relatively short half-life (Zaccara et al., 1988). Moreover, the main older drugs are cleared to a significant extent by biotransformation, and some of them (primidone, diazepam and clobazam) have active metabolites which may complicate evaluation of concentration-response relationships. These contrasts in pharmacokinetic properties, between the old and new, were also observed with the drugs investigated during this study, namely CBZ and levetiracetam. Although, CBZ is regarded as the first line drug in the management of partial seizures, with and without secondary
generalisation, and of generalised tonic-clonic seizures it has some rather unique pharmacokinetic properties which makes its clinical use somewhat difficult. It is extensively metabolised to the active metabolite CBZ-E, which has been shown to possess similar anticonvulsant properties to CBZ (Faigle & Feldmann, 1975; 1989; Faigle et al., 1976; Frigerio et al., 1975; Bourgeois et al., 1984), and may contribute to many of the side effects associated with CBZ therapy (Patsalos et al., 1985; Gillham et al., 1988; Warner et al., 1992). Other metabolites do exist but do not contribute to CBZ’s antiepileptic effect or its adverse side effects, due to their low potency or low concentration, or both (Morselli et al., 1975). Furthermore, CBZ has the unique ability to induce its own metabolism, a phenomenon known as “autoinduction” resulting in a very short half-life. The elimination half-life of CBZ can be up to two to three times longer after the first dose than after 4 weeks of chronic administration (Eichelbaum et al., 1975; 1976; 1984; 1985; Geradin et al., 1976; Bertilsson et al., 1980). The extent of CBZ autoinduction of the metabolizing enzymes, and possibly also heteroinduction by other AEDs, is considered to be responsible for the considerable diurnal variation in serum CBZ concentrations (MacPhee et al., 1987). These unique pharmacokinetic properties were also evident during this investigation. For instance, after reaching an initial steady-state, CBZ induced its own metabolism resulting in a dramatic reduction of both CBZ and CBZ-E concentrations in blood and CSF. Additionally, CBZ-E was detected in both blood and CSF samples, shortly after CBZ. In fact, eventually higher concentrations of the metabolite than the parent drug were observed approximately 5-6 hours after CBZ was first administered. The large fluctuations of CBZ concentrations, mentioned earlier, were also observed during the first day of its administration.

In contrast, levetiracetam seems to possess many pharmacokinetic advantageous features, compared to that of CBZ. The data presented in this thesis show that levetiracetam exhibits linear pharmacokinetics when administered both acutely and chronically. Unlike CBZ, levetiracetam is not extensively bound to plasma proteins. Furthermore, levetiracetam appears to have no active metabolites (Gower et al., 1992; Gower et al., 1995; Noyer et al., 1995), although this was not investigated during this
Overall, levetiracetam appeared to have far simpler pharmacokinetics than that of CBZ, suggesting that it should be simpler to use both experimentally and clinically.

The extent by which an AED is metabolized in the liver is another important consideration. Thus whilst less than 1% of CBZ is excreted unchanged in urine (Bertilsson & Tomson, 1986; Faigle & Feldmann, 1989; Liu & Delgado, 1994), whereas approximately two-thirds of the administered dose of levetiracetam is recovered unchanged in the urine. One quarter is eliminated as an inactive metabolite (Walker & Patsalos, 1995; Perucca & Bialer, 1996). The availability of compounds which are eliminated primarily in unchanged form may be particularly advantageous in the management of epileptic patients suffering from hepatic disease or taking concomitant medications known to interfere with hepatic drug metabolism.

The issue of drug interactions is yet another useful aspect to address when prescribing a drug to abolish epileptic seizures, since AEDs are given for prolonged periods and often in combination. Again the older AEDs have proven to have a high interaction potential. For instance CBZ, phenytoin and the barbiturates, are all inducers of the hepatic microsomal enzymes (Perucca et al., 1984), resulting in acceleration of the metabolism of glucocorticoids, oral contraceptive steroids, coumarin anticoagulants, antidysrhythmic agents and many other drugs (Perucca, 1987). Whereas, it has been suggested that valproic acid may act as an enzyme inhibitor (Steiner et al., 1994), and may displace various drugs from plasma protein binding sites (Perucca et al., 1980). Many of the newer drugs have clear advantages in terms of a lower interaction potential. In particular, gabapentin, vigabatrin, lamotrigine and tiagabine are devoid of enzyme inducing activity (Richens, 1993; Brodie et al., 1995; Perucca & Bialer, 1996). Although clinical experience with levetiracetam is limited we know that it has a low potential for drug interactions. It does not appear to affect serum concentrations of most AEDs, including carbamazepine, phenobarbitone, valproic acid, primidone, clobazam and demethylclobazam. However it has been shown to increase serum concentrations of phenytoin during concomittent therapy in some patients, (Patsalos
et al., 1994; Sharief et al., 1996). In conclusion it would appear that the newly developed AEDs seem to have more ideal pharmacokinetic properties compared to that of their predecessors.

9.3 **Acute and chronic pharmacokinetic profiles of AEDs**

In order for the optimal therapeutic potential of a drug to be achieved, it is essential to devise a rational dosing regime based on its pharmacokinetics. Pharmacokinetic considerations are important both for deciding the initial dosage and therapeutic drug monitoring which is performed routinely most AEDs. The amount of the drug to be given is based on desirable targets or therapeutic ranges. For centrally acting drugs such as AEDs it is assumed that the total serum concentrations reflect the concentrations in the CNS (Goldberg & Crandall, 1978; Tozer & Winter, 1980; Woodbury, 1989). However, most of the studies on which these assumptions are based have been carried on accumulated single sample time points (Reynolds et al., 1972; Houghton et al., 1975; Koren et al., 1983; Brodie et al., 1985; Friel et al., 1989) or, from serial samples of blood or CSF, but rarely both (Frey & Loscher, 1978; Loscher & Frey, 1984; Ramzan & Levy, 1989; Sechi et al., 1989). Therefore there is a growing need for more data on central and peripheral pharmacokinetics of AEDs in individual subjects, in order to establish the relationship between central and peripheral pharmacokinetics of AEDs.

The animal model used in this thesis (Patsalos et al., 1992) to determine the pharmacokinetics and neuropharmacokinetics kinetics of CBZ and levetiracetam, allows simultaneous sampling of blood and CSF. It has already been used to study the AEDs, CBZ, phenytoin and milacemide when injected intraperitoneally (Patsalos et al., 1992; Semba et al., 1993a,b). This model has numerous advantages over other methods namely (a) as serial sampling of blood and CSF can be achieved with minimal handling and induced stress, kinetic parameters are obtained under physiological conditions (b) as each rat serves as its own control, interindividual variability (as occurs when composite values of individual rats killed at different time points after drug administration are used) is minimized and thus the number of
experimental animals needed to obtain detailed kinetic data is reduced and (c) long sampling protocols can be used in determining drug kinetics. Both acute and chronic studies can be undertaken to study the inter-relationship between drug kinetics at central (CSF) and peripheral (blood) sampling sites. It is advantageous to monitor centrally active drugs in a central compartment, each rat serves as its own control and interindividual variability is minimised. Also, results can be combined to give mean values for groups but with the practical advantage that far fewer animals are required than with conventional methodology.

The results of the CBZ chronic pharmacokinetic study emphasizes the importance of determining both the peripheral and central kinetics of a drug using a model which allows concurrent sampling over an extensive period. Since it allowed a more quantitative assessment of the process of autoinduction. This model allowed clear differentiation of the relationship between blood/CSF CBZ/CBZ-E concentrations and an antiepileptic effect. Unfortunately due to the physical properties of CBZ (highly insoluble in water) and the osmotic minipump (limited reservoir volume) it was not possible to investigate the effects of higher CBZ doses. In addition, because of the use of the osmotic minipump the model used in this thesis entailed an additional surgical procedure, but fortunately this did not seem to influence seizure frequency in the tetanus toxin model. A simpler pharmacokinetic profile was observed during the chronic levetiracetam pharmacokinetic study. Once steady-state was achieved levetiracetam concentrations remained constant in both the serum and CSF compartments. These concentrations were linearly related to the antiepileptic effects exhibited by levetiracetam in the tetanus toxin model of epilepsy.

The use of the model, however, requires good surgical expertise, thus although the overall success rate of the blood and CSF cannulation was quite high, the patency of both the blood and CSF catheters decreased with time. Consequently, the number of blood and CSF catheters patent by the end of the chronic study (71% and 57%, respectively) was far less than that of the acute study (88% and 88%, respectively). The biggest problem was trying to prevent the animals from removing their catheters,
as this accounted for the largest amount of failures for both blood and CSF catheters (58 % and 44 %, respectively). The reason why less CSF catheters remained patent (57 %) by the end of the chronic study compared to the number of blood catheters (71 %), can probably be explained by the fact that the dental cement used to secure the CSF catheter caused more irritation to the animal, than the tubing used with the blood cannulation.

In conclusion, although some degree of expertise was required for the surgery, this pharmacokinetic model proved to be easy to use, less expensive than the conventional models and reliable, with minimal interindividual variability observed.

9.4 Assessment of AEDs in a chronic disinhibition model

The ultimate test for any drug is to see if it can achieve its desired affect. In the case of an AEDs an obvious question to ask is whether seizures can be controlled, or even better, abolished by antiepileptic medication. While the assessment of potential antiepileptic agents in humans is fraught with methodological and ethical dilemmas, such assessment in animal models of epilepsy poses fewer problems.

The choice of a particular model will depend critically on the nature of the study, the questions posed and hence the kinds of samples, recordings or observations required. Acute models such as pentylenetetrazol (PTZ; Marescaux et al., 1984; Piredda et al., 1985; Santucci et al., 1985) and the maximal electroshock (MES; Berman & Alder, 1984; Loscher et al., 1986; Fisher, 1989; Redgrave et al., 1992; Mulzac & Scott, 1993) are extremely useful for the extensive and effective screening of, and preliminary identification of AEDs. These models have provided a significant insight into epilepsy at the cellular level. They have provided us with a means to search for the fundamental mechanism underlying epileptic discharges. However, despite the advantages of these acute models, in particular the brain slice preparation maintained in vitro, notably in the stability and ease of recording, the hippocampal slice has its limitations, notably the absence of the rest of the brain. Therefore, acute models can only be used to assess specific symptoms, e.g. interictal spikes or seizures, rather than
epileptic states. Chronic models, however produce a prolonged state of increased seizure susceptibility, or to recurrent spontaneous seizures. Chronic experimental epilepsies can be compared more closely with clinical conditions than can acute models. They are better suited for behavioural and EEG studies in freely moving animals. The study of potential AEDs has largely been dependent upon the use of both acute and chronic animal model systems. If such drugs prove successful in the experimental epilepsy then it should be possible to investigate further the possible mode of action of these drugs.

The tetanus toxin induced epilepsy model provides a particularly useful model for the study of seizures, in particular complex partial seizures. It allows the investigator to study the seizure syndrome and the nature of any behavioural abnormalities during the seizure course. The seizures and other epileptic activity, such as inter-ictal and polymyseps, are spontaneous and recur intermittently over a period of several weeks (Mellanby et al., 1977, 1981; Hawkins, 1987). The model is therefore of epilepsy and not just of seizures. Additionally, it provides the investigator the opportunity to study the progression of recurrent seizure activity at the levels of the EEG, behaviour, cellular physiology, histology and molecular biology. This model has been extensively studied by Mellanby and Hawkins. Therefore, this project dealt very little with the characterisation of this animal model of epilepsy. Nevertheless, an attempt to further elucidate and validate this model was made by investigating the effect of chronic CBZ and levetiracetam via an osmotic minipump on seizures. Numerous AEDs, namely CBZ, phenytoin, valproic acid, piracetam and oxiracetam, have been studied previously in this chronic epileptic model. Carbamazepine (20, 40 and 60 mg/kg, given orally) was found to be effective by reducing the maximum number of seizures occurring on one day, and with the highest dose, a significant reduction in the total number of seizures (Hawkins et al., 1985). This effect was later shown to be potentiated by the nootropic drug piracetam (Hawkins et al., 1986). Phenytoin and sodium valproate, however were found to be ineffective at reducing the number of seizures, although they did reduce their severity (Mellanby et al., 1985).
A similar pattern was observed to that of Hawkins and Mellanby in relation to CBZ. A statistically significant reduction in seizures was observed, before autoinduction occurred, with the number of generalised seizures occurring on one day. During the process of autoinduction seizures gradually returned and were eventually as prominent as in the control group. However, in contrast to Hawkins et al., (1986), a statistically significant result was not achieved at the higher dose with the total number of generalised and non-generalised seizures occurring throughout the syndrome despite achieving similar CBZ blood concentrations. As a result it did not seem necessary to investigate the effect of the lower dose of CBZ. This contradicts the findings of Hawkins & Mellanby, 1986, who observed a significant reduction in the total number of seizures occurring throughout the syndrome. These differences may stem from the different experimental conditions used, such as the mode of administration, the time period studied, the dosages used, and the statistical analysis performed on the results.

Constant daily observation and handling of the animals showed that any antiepileptic effect observed, was not associated with obvious adverse effects such as sedation and nausea. From the initial clinical studies with CBZ it was observed that the drug did not normalise the EEG (Wilkus et al., 1978). Likewise, from general observation of the electrographic traces of animals in which the EEG had been continuously recorded from, post and pre- CBZ administration, the interictal spikes still remained and no difference in frequency was observed. In fact they were as prominent if not more so in some animals, during CBZ medication then they were before CBZ administration had begun.

Levetiracetam, administered continuously, at a dosage of 8 and 16 mg/kg also had an antiepileptic effect in the tetanus toxin epilepsy model. With the lower dose, the reduction in the total number of generalised and non-generalised seizures (39.1 and 35.8 %, respectively) was statistically insignificant (p=0.071 and p=0.635, respectively). With the higher dose, however, there was a significant reduction in the total number of generalised and non-generalised seizures (56.7 and 41.2 %,
respectively), suggesting that there may be a dose response relationship. This reduction was statistically significant with the generalised but not with the non-
generalised seizures (p<0.05 and p=0.525).

Probably the most common adverse consequences of the tetanus toxin was a change in the behaviour of the animal. The toxin injected animals became increasingly more difficult to handle, reacted with aggression to any attempt at handling and jumped high in response to nearly every stimulus. The animals having received a buffer injection, quickly became perfectly normal and indistinguishable from naive rats after the operation, suggesting that this behavioural change was not due to the surgical procedure itself. Although no exact measurement was taken it was observed that the degree of aggressive behaviour was directly associated with the frequency and severity of the seizures the animal was having. The more frequent and the worse the seizures were the more hostile the animal became. Due to this aggressiveness, dosing animals up to three times a day was not feasible, as the animal became increasingly irritated and was more likely to have a seizure when handled. Undoubtedly this would have affected the results of this study. Therefore, to minimise the amount of stress to both the animal and handler the use of the minipump as the mode of administration of the drugs proved to be exceptionally beneficial here. The animal only needed to be handled on one occasion for implantation of the minipump (and the drug was continuously delivered at a constant rate for seven days, thereafter).

One disadvantage of the model, perhaps, was the need for a second surgical procedure so as to implant the osmotic minipump. This procedure required the animal to be anaesthetised a week after the injection of the tetanus toxin. However, this implantation procedure had little, if any, adverse effects on the animals. The minipump did not appear to cause the animals any discomfort in their movement around the cage. The survival rate for the acute and chronic pharmacokinetic study was 86.5 %. Of the remaining 13.5 % that died, 42.96 % died due to respiratory distress. Very little could be done to improve this statistic. No animal died as a direct result of the implantation of the minipump. Most other deaths could probably be
attributed to inexperience of the investigator in carrying out the surgical procedures.

In summary the tetanus toxin model’s strength and weaknesses both stem from spontaneous, intermittent occurrence of electrographic seizures over prolonged periods. The major advantage is that this model models not only an epileptic seizure but also epilepsy as a chronic progressive disorder. The disadvantage is that it is technically difficult to score epileptic activity and measuring the incidence and severity of the epileptic attacks is extremely time consuming, as they occur spontaneously and rather irregularly. However, the in vivo model always has closer parallels with clinical epilepsies than in vitro models and this one is no exception to the rule.

9.5 Assessment of AEDs in an acute disinhibition model

The second animal model used in this investigation was the in vitro hippocampal slice taken from naive rats. The epileptic syndrome was instigated by modification of the ACSF. In this instance the slices were bathed in bicuculline and raised concentrations of extracellular potassium. This disinhibition model proved to be successful in the study of seizure-like events in vitro. Similarities of electrographic traces recorded in vivo and in vitro were observed suggesting that the hippocampal slice preparation not only preserves the basic physiology of the hippocampus, but also mirrors epileptic conditions realistically, when compared to in vivo models. Moreover, the similarities between the secondary bursts and the rhythmic bursts in vivo make similar underlying mechanisms rather likely. Levetiracetam exerted antiepileptic properties in both epileptic models used, in a similar manner. The antiepileptic effect of levetiracetam observed in the in vivo model resulted in a reduction in the number of generalised seizures over the period investigated. Generalised seizures differed electrographically from non-generalised seizures in many respects but in particular generalised seizures were often associated with stage 5 rhythmic interictal bursts. It was suggested in Chapter 8 that these rhythmic bursts were the equivalent to the secondary bursts recorded in vitro. The antiepileptic effect of levetiracetam on the seizure-like events recorded in vitro was demonstrated by a
significant dose-dependent reduction in the length of these epileptiform discharges. Levetiracetam had no effect, however, on the general pattern of the seizure-like events. If the rhythmic interictal bursts of stage 5 are indeed equivalent to the secondary bursts recorded in vivo, these data suggest that levetiracetam may be acting on the same mechanism responsible for these discharges, in both models.

9.6 Conclusion

The objective of this thesis was to test the efficacy of the conventional drug CBZ and the putative drug, levetiracetam, on electrographic seizures in animals previously injected with tetanus toxin, and to evaluate the antiepileptic effect of levetiracetam on bicuculline induced epileptiform bursts in the hippocampal slice. The pharmacokinetics and neuropharmacokinetics following acute and chronic i.p. administration of levetiracetam via the osmotic minipump were also determined. A similar kinetic study was performed for CBZ and CBZ-E, following the chronic administration of CBZ. The strategy was to attempt to unravel the basic mechanisms of both antiepileptic drugs, but in particular, levetiracetam.

The results from the kinetic studies of both drugs were very different. CBZ exhibited very complex and undesirable pharmacokinetics, whilst, levetiracetam showed simple, predictable and ideal pharmacokinetics. Consequently these data would suggest that levetiracetam should be simpler to use both clinically and experimentally, than its older predecessor, CBZ.

The results from the antiepileptic studies demonstrated that both CBZ and levetiracetam, administered continuously via an osmotic minipump, exerted a significant antiepileptic effect in rats with chronic limbic epilepsy, induced by injecting tetanus toxin unilaterally into one hippocampus. The antiepileptic effect involved a reduction in the maximum number of both types of seizures occurring on any one day, and a reduction in the total number of generalised seizures. However, a statistically significant result was only achieved following administration of the highest dose of levetiracetam. A significant reduction in overall duration of
generalised seizures was also observed following administration of either drug. In addition levetiracetam was effective in reducing epileptiform activity in rat hippocampal slices, induced by bicuculline and raised extracellular potassium concentrations. This epileptiform activity took the form of trains of population bursts with a distinctive biphasic pattern lasting several seconds. In this model levetiracetam reduced the overall length of the seizure-like events without influencing the biphasic firing pattern. These observations suggest that whereas levetiracetam does not effect epileptogenesis per se it does reduce seizure severity and, particularly, seizure generalisation in vivo in predominantly disinhibitory models.

In conclusion this thesis demonstrates that levetiracetam significantly reduces seizure generalisation in vivo and attenuates prolonged ictaform discharges in the disinhibited hippocampal slice which resemble depth EEG recordings during generalisation. Finally, levetiracetam demonstrated no obvious adverse neurotoxic side effects and simple pharmacokinetics, suggesting it may play an important role in the future treatment of epilepsy.
REFERENCES


CHEVALIER Y., GRANT R. & SANDER-JWAS (1995) Twelve week add-on, increasing dose (1,000-4,000 mg/day) multicenter pilot study of ucb LO59 in epileptic patients. Epilepsia 37, 153(Abstract)


DREIER J.P. & HEINEMANN U. (1991) Regional and time dependent variations of


HAWKINS C.A., MELLANBY J. & BROWN J. (1985) Antiepileptic and


NEBERT D.W., NELSON D.R., COON M.J., ESTABROOK R.W., FEYEREISEN


RAMZAN I. & LEVY G. (1989) Relationship between concentration and anticonvulsant effect of phenytoin against electroshock-induced seizures in rats:


SEMBa J. & PATSALOS P.N. (1993) Milacemide effects on the temporal


APPENDIX 1

As the tetanus toxin model had been extensively characterised by Mellanby et al., 1977; 1981; 1984 and Hawkins et al., 1985, no attempt was made to re-characterise it for use in this thesis. Although many aspects of this model were similar to that of Hawkins et al., 1985, some differences did pertain. Such differences included:

(i) mode of drug administration: oral administration, three times a day (Hawkins et al., 1985) versus continuous infusion via the minipump (this thesis)
(ii) the time period studied; several weeks (Hawkins et al., 1985) versus one week (this thesis) and
(iii) the tetanus toxin injection site; bilateral (Hawkins et al., 1985) versus unilateral injection (this thesis) and ventral (Hawkins et al., 1985) versus dorsal hippocampus (this thesis).

When investigating CBZs/CBZ-Es antiepileptic effects on seizure frequency in the tetanus toxin model during this thesis, CBZ and CBZ-E did reduce the number of generalised and non-generalised seizures, following continuous intraperitoneal infusion of the drug (4mg/kg/h). However, this reduction was statistically insignificant. In contrast Hawkins et al., 1985 found that with their highest dose (60 mg/kg) administered orally three times a day, there was a statistically significant reduction in the total number of overt seizures. The difference in results is probably due to a combination of the mode of drug administration and the time period studied.

The CBZ concentrations measured by Hawkins et al., 1985, were within the therapeutic range for CBZ, as defined for man and indeed were comparable to those observed in this thesis. However, these concentrations only pertained prior to autoinduction. Additionally, although Hawkins et al., 1985 did not measure the amount of CBZ-E concentrations, the authors suggested that measured CBZ concentrations represented only a proportion of the concentration of active drug, as it had been previously proven that the plasma concentrations of CBZ-E is equal to or greater than that of CBZ (Faigle & Feldmann, 1982). Therefore it may be concluded that due to the mode of administration used in this thesis, the CBZ/CBZ-E concentrations achieved were too low to have any substantial antiepileptic effect on

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the seizure frequency. Unfortunately due to a combination of the physical properties of CBZ and the osmotic minipump it was not possible to increase the dosage of CBZ. This lack of effect of CBZ on seizure frequency in the same epilepsy model emphasizes the clinical advantages and importance of determining the pharmacokinetics of a drug, as it allows a more quantitative assessment of the potency of the drug being administered.

Finally injection of tetanus toxin either bilaterally into the ventral hippocampi or unilaterally into the dorsal hippocampus, both caused a chronic focal epileptiform syndrome in which the animals had intermittent seizures, electroencephalographic signs of epilepsy and a wide range of behavioural abnormalities. Additional similarities in the results of the two different protocols mainly involved the pattern of the seizure syndrome. For instance, the onset of the seizures was rapid, with interictal spikes, polyspikes, and seizures, all occurred spontaneously on a recurrent basis with non-generalised seizures occurring more prominently than generalised seizures at the beginning of the seizure syndrome. However, differences which may be attributed to the injection site were observed and mainly involved the general behaviour of the animal. During the course of the seizure syndrome the animals used during this thesis became increasingly irritable and hyper-reactive with minimal provocation. As the seizure course progressed and the number of seizures increased, the animals became less tolerant and resorted to biting if any attempt was made to handle them. This suggested that the abnormal behaviour was a consequence of the seizures rather than the effect of the toxin itself. However, the animals used by Hawkins et al., 1985 were found to be hyper-reactive and aggressive several days before seizures were first witnessed, implying no apparent correlation between seizures and abnormal behaviour. The differences between our data and that of Hawkins et al., 1985 thus suggest that there may be behavioural difference to epilepsies originating in either the ventral or dorsal hippocampi. Also, as CBZ had an insignificant antiepileptic effect on seizure frequency with our animals compared with a significant effect with the animals of Hawkins et al., 1985 this could imply that CBZ may be more potent on seizures originating in the ventral hippocampus.