To Martin, thank you for those "bankers"!

With best wishes

Patrick

Refractory Epilepsy: Natural History and Pathogenesis

A thesis by

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List of commonly used abbreviations

ABC	ATP-binding cassette
AED	Antiepileptic drug
AGS	Audiogenic seizures
AVM	Arteriovenous malformation
BBB	Blood-brain barrier
BDZ	Benzodiazepine
CBZ	Carbamazepine
CD	Cortical dysplasia
CNS	Central nervous system
CT	Computed tomography
DEPC	Diethylpyrocarbonate
ESM	Ethosuximide
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EEG	Electroencephalography
GABA	γ-aminobutyric acid
GAD	Glutamic acid decarboxylase
GBP	Gabapentin
GEPR	Genetically epilepsy-prone rat
GTCS	Generalised tonic-clonic seizures
HS	Hippocampal sclerosis
IGE	Idiopathic generalised epilepsy
ILAE	International League Against Epilepsy

ISC	Internal standard control RNA
LTG	Lamotrigine
MDR	Multidrug resistance
MMLV	Moloney-murine leukaemia virus
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MTLE	Mesial temporal lobe epilepsy
MTS	Mesial temporal sclerosis
OXC	Oxcarbazepine
PB	Phenobarbital
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PHT	Phenytoin
RNA	Ribonucleic acid
rNTP	Ribonucleoside triphosphate
RT	Reverse transcription
RT-PCR	Reverse transcriptase-polymerase chain reaction
TGB	Tiagabine
TPM	Topiramate
VPA	Sodium valproate/valproic acid
VGB	Vigabatrin

Summary

Despite antiepileptic drug (AED) treatment, up to one third of patients continue to have seizures. Refractory epilepsy is a poorly understood subject, both in terms of its development and pathogenesis. Outcome studies have focused on terminal remission, but little is known about the natural history of epilepsy in terms of its progression to eventual remission or persistent refractoriness. Such an understanding is essential to the formulation of a rational management approach.

Natural history of treated epilepsy

Long-term outcome of newly diagnosed patients was investigated longitudinally for up to 15 years. Response to the first drug and successive substitution monotherapy or polytherapy was analysed. Outcome among patients with different neuropathologies was compared in a separate study.

Among 470 newly diagnosed patients, 64% became seizure-free for at least a year. Patients with high numbers of pre-treatment seizures were less likely to become seizure-free. Epilepsy was controlled by the first AED in 47% of patients. Patients with symptomatic or cryptogenic epilepsy were less likely to become seizure-free on the first AED, partly because they were more likely to develop intolerable side-effects compared to those with idiopathic epilepsy. The majority of such withdrawals occurred at low doses of the three most commonly prescribed AEDs, carbamazepine (CBZ), sodium valproate (VPA) and lamotrigine (LTG). Over 90% of seizure-free patients also required only a moderate daily dose (up to 800mg CBZ, 1500mg VPA, 300mg LTG).

The probability of attaining seizure-freedom declined progressively with successive AED regimens. While 47% became seizure-free on the first drug, only 10% did so on the

second drug and 1% on the third monotherapy. Three percent became seizure-free on a combination of two AEDs. The subsequent seizure-free rate was 41% among those failing the first drug due to intolerable side effects, but only 11% among those in whom the first AED was well tolerated but did not control the seizures completely.

Among patients with inadequate seizure control on the first well tolerated AED, those who received substituted monotherapy and those who received add-on treatment had similar seizure-free rates and incidence of intolerable side effects. More patients became seizure-free on combinations involving an AED that blocked sodium channels and one with multiple mechanisms of action than on other combinations. Combination treatment was more effective when prescribed immediately after the first drug failed due to inadequate seizure control than when it was delayed until a substitution also proved unsuccessful.

In a separate study, compared with other pathologies identified on magnetic resonance imaging, mesial temporal sclerosis (MTS) was associated with the worst prognosis, although 42% did become seizure-free on AED treatment.

Pathogenesis of refractory epilepsy

Two candidate biological mechanisms in the pathogenesis of refractory epilepsy were studied. Glutamic acid decarboxylase (GAD) autoantibody titres were compared between patients with controlled and uncontrolled epilepsy. GAD catalyses the conversion of glutamate to γ-aminobutyric acid, the major inhibitory neurotransmitter. Autoantibodies against GAD are prevalent in insulin dependent diabetes mellitus, and have been documented in anecdotal cases of refractory epilepsy.

The drug transporter P-glycoprotein (P-gp) was investigated in a series of laboratory-based pilot experiments. Encoded by the multidrug resistance gene family (*MDR1* in man and *mdr1a* and *1b* in rodents), P-gp actively extrudes a wide range of xenobiotics out of cells. Its over-expression is thought to underlie the resistance of some cancers to multiple chemotherapeutics. P-gp is physiologically expressed at high level in the cerebral capillary endothelium where it contributes to the integrity of the blood-brain barrier. Over-expression of P-gp in brain tissues resected from patients with refractory epilepsy has been reported in surgical case series.

Mdr1a(-/-) mice devoid of cerebral P-gp were used to determine whether AEDs were substrates of the drug transporter. The pharmacokinetic profiles of four established and four new AEDs in *mdr1a*(-/-) mice and wild-type mice were compared.

The technique of quantitative reverse transcriptase-polymerase chain reaction was developed and validated to determine tissue concentration of *mdr1* mRNA as an indicator of gene expression. Expression was determined in different regions of the normal rat brain, and in brains of genetically epilepsy-prone rats (GEPRs) subject to a single audiogenic seizure. To explore the effect of tissue damage, a laser beam was impinged upon the cerebral cortex of rats. *Mdr1* expression was measured in tissues surrounding the focal necrosis. Human brain tissues resected during epilepsy surgery were also analysed for *MDR1* gene expression.

There was no difference in GAD autoantibody titres between patients with controlled and uncontrolled epilepsy. Four female patients with uncontrolled epilepsy had elevated antibody titres, but three of them also tested positive for pancreatic islet cell antibodies, suggesting a possible pancreatic source of GAD antibodies.

Topiramate, phenytoin and CBZ reached higher brain concentrations in *mdr1a*(-/-) mice compared to wild type mice, suggesting they are substrates of P-gp. *Mdr1b* was expressed to an appreciable level in the hippocampus alone, whereas *mdr1a* was expressed uniformly throughout the normal rat brain. After a single seizure, *mdr1a* expression became elevated at 4 hours in the cerebral cortex of GEPRs, rising to a maximum at 24 hours, returning towards basal level at 7 days. Similar time-dependent changes were observed in the midbrain, but *mdr1a* expression remained unchanged in the pons/medulla and hippocampus. Focal laser application induced a transient expression of *mdr1b* in the rat cerebral cortex at 1 week. Significant *MDR1* expression was detected in brain tissues resected from two patients who underwent temporal lobectomy for refractory epilepsy.

Conclusions

Patients with symptomatic epilepsies, particularly those with MTS, and patients with inadequate response to initial treatment are likely to develop refractory epilepsy. Nearly 50% of newly diagnosed patients responded to the first AED, with over 90% doing so at moderate dosing. Tolerability was an important factor in determining the overall effectiveness of AED treatment. Over-expression of P-gp in the hippocampus, as a result of recurrent seizures, might contribute to the pathogenesis of refractory epilepsy (in particular temporal lobe epilepsy) by limiting AED access to the seizure focus. A working hypothesis regarding early targetting of rational combination therapy or surgery to "high risk" patients to prevent the progression to the refractory state is proposed.

PART I

OVERALL INTRODUCTION

1 Epilepsy

"Epilepsy" is derived from the Greek word *epilambanein*, meaning "to seize" or "to attack". It is the most common serious neurological disorder. Between 40 and 70 per 100000 people develop epilepsy each year. Most epidemiological studies report a point prevalence between 4 and 10/1000 (Hauser et al, 1991; Cockerell et al, 1995a; Sander and Shorvon, 1996). Epilepsy crosses geographical boundaries. Higher incidence and prevalence rates are reported in developing countries, possibly due to more widespread parasitic diseases (Lavados et al., 1992; Placentia et al., 1992). Even excluding febrile convulsions, which occur in about 5% of children (Nelson and Ellenberg, 1976), it is estimated that as many as 8.2% of the population will have a seizure at some point in their lives (Hauser et al, 1996). Epilepsy affects all ages, with high rates occurring in early childhood, falling to low levels in early adult life, but rising again in those over 55 years. In the Rochester study, the highest incidence of epilepsy is found in those aged 75 years or older (Hauser et al, 1996).

Epilepsy transcends history. Seizures have been recorded by all races and creeds since the dawn of literacy. They were formally recognised in the classical Chinese medical text, *Huang Di Nei Ching* (The Yellow Emperor's Classic of Internal Medicine), written around 770-221 B.C. (Lai and Lai, 1991). In the West, the oldest written account of epilepsy is perhaps found in a Babylonian treatise discovered in South Turkey, which ascribed it as "the result of possession by a demon or departed spirit" (Kinnier Wilson and Reynolds, 1990).

The stigmatisation of epilepsy is deep-rooted. In ancient cultures, epileptics were considered unclean or evil, and an epileptic fit constituted a bad omen. The Romans called epilepsy the *morbus comitialis*, attributing the name to the belief that an epileptic attack

would spoil the day of the *comitia*, the assembly of the people. Even physicians advised spitting upon seeing an epileptic in order to "throw back contagion" (Tempkin, 1971). Galen's idea (130-220 A.D.) of "animal spirits" in body tubes was still being quoted by Willis in the late 17th century (Stevens, 1973), and Albich in the 18th century warned "neither talk nor bathe with them (epileptics), since by their mere breath they infect people" (Tempkin, 1971). Even today, although generally to a lesser degree, society's prejudice and ignorance is still felt by many patients (Baker et al, 2000), not least fuelled by often distorted and sensationalised depiction of seizures in the media (Kerson et al, 1999; Krauss et al, 2000) and literature (Vanzan Paladin, 1995).

The first attempt to dispel superstitious and magical beliefs and explain epilepsy rationally was found in the Hippocratic collection of medical writings from about 400 B.C. What the author wrote in the introduction to the book *On the Sacred Disease* may still be applicable today:

> "I do not believe that the so-called 'Sacred Disease' is any more divine or sacred than any other diseases. It has its own specific nature and cause; but because it is completely different from other diseases men through their inexperience and wonder at its peculiar symptoms have believed it to be of divine origin." (Longrigg, 2000)

In the author's opinion, epilepsy is "hereditary", its cause lies in the brain from where phlegm overspills into the blood stream, producing a variety of symptoms depending on the organs affected (Tempkin, 1971). Distinction was made between tonic and clonic seizures, and changes in pulse and respiration were noted. By the time of the second century, seizures were further classified according to their clinical manifestation, with the key central theme being alteration of consciousness and amnesia of the ictal event (Gross,

1992). The term "aura", still widely used today to refer to warning symptoms in general, was introduced by Galen to denote a particular sensation that was described to him as a "cold breeze" by one of his patients. Various provoking factors were recognised, such as alcohol, pregnancy (eclampsia) and head trauma (Gross, 1992).

The development of a rational understanding of epilepsy took a step back in the Middle Ages. The "falling sickness" was believed to be the result of divine or demonic possession, and the variety and range of signs were interpreted according to the different views of supernatural forces prevalent at the time (Gross, 1992). Extraterrestrial influences were evoked. Theological interpretations were particularly prevailing, drawing examples from the Gospels of Mark and Luke which recorded Jesus driving out "unclean spirit" from a boy suffering from convulsions.

Renaissance saw a re-thinking of the views towards epilepsy and a re-emphasis on objective observation rather than dogma. The rise in anatomy allowed the recognition that epilepsy might be associated with other disorders, including syphilis, measles, making the major leap in understanding that seizures could be a symptom of other diseases. "Minor", "medium" and "major" seizures were recognised by their signs and symptoms, and obstruction of arteries, nerves or ventricles was thought to be the cause. This humoral theory was finally abandoned when Le Pois found no evidence of ventricular obstruction but observed local "irritation" in postmortem examinations (Gross, 1992). Thus the foundation was laid for the modern scientific approach to the understanding of epilepsy that began during the Enlightenment.

The rise of the disciplines of anatomy, chemistry, pathology and physiology allowed the development of rational pathophysiological hypotheses of seizures and epilepsy. Epilepsy

and hysteria were distinguished. The association with the phases of the moon was scientifically disproved by independent prospective observations by Leuret and Moreau (Tempkin, 1971), notwithstanding today's interest in catamenial epilepsy, which was also noted in the early 18th century by Maisonneuve (Esquirol, 1845). Absence, partial and generalised seizures and status epilepticus were coined in the new terminology. Aided by advances in anatomy and the emerging view of cerebral localisation, various intracranial abnormalities were recognised to cause epilepsy, which was classified as "idiopathic". Work by Todd and Bright dispelled the notion of "sympathetic" epilepsy due to peripheral irritation and demonstrated that diseases within the brain could also be responsible for focal seizures (Gross, 1992). Tissot further provided a framework from which the present day classification systems were loosely derived (Dreifuss, 1997). Epileptic seizures were classified and distinguished from the underlying causative conditions that we now call the epilepsies. An epilepsy was noted to be composed of an ongoing predisposing condition but individual seizures were the expression of the epileptic process, the presenting symptom thereof.

The modern chapter of epilepsy began when the Yorkshireman John Hughlings Jackson linked these clinical observations with new electrophysiological data that showed that electrical stimulation of discrete areas of cortex elicited specific motor effects (Fritsch and Hitzig, 1870). He was able to formulate a complete pathophysiological model of epilepsy in his *Investigations of Epilepsies* (1873), asserting that:

> "A convulsion is but a symptom, and implies only that there is an occasional, an excessive, and a disorderly discharge of nerve tissue on muscles."

Jackson believed that epilepsy was due to a paroxysmal discharge of neurones within a local area that could then affect more normal areas of the brain. Here, finally, was a statement that linked the diverse symptomatology of seizures to their physiology: "According as the seat of the discharge varies the symptoms of the paroxysm vary." All seizures, therefore, have a similar pathophysiology - a hypothesis still actively investigated.

Objective evaluations of the epilepsies ensued. Gowers' elegant description of seizures around the turn of the century was remarkable in its detail and depth. He discussed his cases in light of recent physiological advances and emphasised the relationship of partial seizures to focal lesions and the lack of focal pathology in cases of generalised seizures (Gowers, 1881). In the 1930s, Berger discovered typical electroencephalographic (EEG) abnormalities in patients with epilepsy. This, together with the work of Penfield and Jasper (1954), who reported a relationship between the functional anatomy of the brain and the specific nature of ictal activity, laid the foundations of the present understanding and classification of epilepsy.

1.1 Definitions

As eluded above, epilepsy is not a single disease entity. Rather, the generic term refers to a collection of conditions with a wide range of underlying aetiologies and pathologies, all with the common and fundamental characteristic of recurrent, usually unprovoked epileptic seizures (Engel and Pedley, 1997). Strictly speaking, epilepsy is a mere symptom of the underlying aetiology. A person is said to have epilepsy if he/she is prone to experiencing recurrent seizure. This definition invites uncertainty, as in marginal cases it may be difficult to clearly define a liability to future attacks. In most practical situations epilepsy is diagnosed when two or more unprovoked seizures have occurred.

An epileptic seizure is defined as the manifestation of an abnormal and excessive synchronised discharge of a set of neurones in the brain. The clinical features consist of a wide range of sudden and transitory abnormal phenomena which may include alterations of consciousness, motor, sensory, autonomic, or psychic events (Commission, 1993). By this definition, subclinical attacks in which epileptic discharges detectable on EEG but not accompanied by evident symptoms or signs are not considered to be epileptic seizures. A given patient may exhibit more than one type of seizures.

Separate systems have been proposed by the International League Against Epilepsy (ILAE) to classify epileptic seizures and epileptic syndromes.

1.2 Classification of seizures

Since seizures are by definition a symptom rather than a distinct disease, their classification is necessarily arbitrary and phenomenal. The present commonly used classification was proposed by the ILAE nearly twenty years ago (Commission, 1981) using clinical description and EEG characteristics (Table 1). With the advances in imaging and neurophysiology over the years, the limitations of the present classification are becoming increasingly apparent. According, it is under current review. A seizure classification based exclusively on ictal semiology has been advocated to avoid confusion with the classification of epileptic syndromes (Lüders et al, 1998). Nevertheless, the present system has gained widespread acceptance and has provided an effective means of communication among clinicians.

Table 1. International classification of epileptic seizures*

I	**Partial (focal) seizures** (seizures beginning locally)		
	A	Simple (consciousness not impaired)	
		i	with motor symptoms
		ii	with somatosensory or special sensory symptoms
		iii	with autonomic symptoms or signs
		iv	with psychic symptoms
	B	Complex (with impaired consciousness)	
		i	simple partial onset followed by impairment of consciousness
		ii	impaired consciousness at onset
	C	Partial seizures evolving into secondary generalised seizures	
II	**Generalised seizures** (convulsive or non-convulsive)		
	A	Absence seizures	
		i	typical (petit mal)
		ii	atypical
	B	Myoclonic seizures	
	C	Clonic seizures	
	D	Tonic seizures	
	E	Tonic-clonic seizures (grand mal)	
	F	Atonic seizures	
III	**Unclassified seizures**		

*Adapted from Commission, 1981.

The current classification divides seizures broadly into partial and generalised. Partial seizures originate in a focal region of the cortex in one hemisphere and are subdivided into those that occur without alteration of consciousness (simple partial) and those in which consciousness is impaired (complex partial). The behavioural manifestations of a simple partial seizure are determined by the functions normally served by the cortical region from which the seizure arises. The majority of complex partial seizures originate from the temporal lobe. Both types of partial seizures can spread rapidly to other cortical areas through neuronal networks, resulting in secondary generalised tonic-clonic seizures.

Generalised seizures are characterised by widespread cortical and subcortical involvement at the outset and consciousness is usually impaired. They are accompanied by bilateral, symmetrical synchronous EEG discharges. An absence seizure (petit mal) is characterised by an abrupt cessation of ongoing activities associated with a blank stare lasting a few to 30 seconds and followed by an abrupt return to normal behaviour. An atypical absence seizure lasts longer and is often associated with changes in muscle tone. A myoclonic seizure consists of brief, shock-like muscle contractions that may occur singly or in clusters, and can affect any group of muscles. Recovery is immediate and the patient often maintains that consciousness was not lost. A tonic seizure consists of sustained muscle contractions, whereas muscle contractions are rhythmic and irregular in a clonic seizure. Tonic-clonic seizures (grand mal) are the form of attack most recognised by clinicians and the general public alike. There is sudden loss of consciousness and falling to the ground, often preceded by a cry. The initial tonic phase, typically lasting 10-30 seconds, is followed by bilateral symmetrical convulsive movements, sometimes with tongue biting and urinary incontinence. Post-ictally the patient is confused and often lapses into deep sleep, returning to normal minutes or hours later. In an atonic seizure, there is a sudden

loss of muscle tone, which can be generalised causing collapse, or restricted to certain muscle groups (Dreifuss, 1997).

1.3 Classification of epilepsy

The assignment of seizures to a particular category is largely observational and does not accurately reflect such characteristics as underlying aetiology, anatomical site of seizure-focus, prognosis and response to treatment. A separate classification system of epilepsies and epileptic syndromes was proposed by the ILAE in 1985 and revised in 1989 (Commission, 1989), which takes into account the characteristics of the seizures, EEG changes, age of onset, family history, etc (Table 2). Epilepsies are separated into generalised and localisation-related (focal, partial) according to the mode of onset of seizures, and each is further subdivided into idiopathic, symptomatic and cryptogenic based on the knowledge of aetiology. Idiopathic epilepsies are "not preceded or occasioned by another", and are presumed to be genetic in origin. Symptomatic epilepsies are considered the consequence of a known cerebral abnormality, such as mesial temporal sclerosis (MTS), cortical dysplasia, arteriovenous malformation, stroke, or cerebral palsy. Cryptogenic epilepsies are presumed to be symptomatic but with unidentified underlying abnormality on the basis of clinical information and investigation results.

While the full version of the classification is complex (and perhaps confusing), categorising a patient's epilepsy into idiopathic, symptomatic or cryptogenic does give broad indication of possible underlying aetiology and can have implications for management and prognosis. Making an accurate diagnosis of an epileptic syndrome, particularly the idiopathic epilepsies, may allow the clinician to define the likely prognosis, provide appropriate genetic counselling and choose the most appropriate AED therapy (Brodie and French, 2000).

Table 2. International classification of epilepsies and epileptic syndromes*

1 **Localisation-related** (focal, local, partial)
- 1.1 Idiopathic (primary)
  - Benign childhood epilepsy with centrotemporal spike
  - Childhood epilepsy with occipital paroxysms
  - Primary reading epilepsy
- 1.2 Symptomatic (secondary)
  - Chronic progressive epilepsia partialis continua of childhood
  - Seizures with specific modes of precipitation
  - Temporal lobe epilepsies
  - Frontal lobe epilepsies
  - Parietal lobe epilepsies
  - Occipital lobe epilepsies
- 1.3 Cryptogenic

2 **Generalised**
- 2.1 Idiopathic
  - Benign neonatal familial convulsions
  - Benign neonatal convulsions
  - Benign myoclonic epilepsy in infancy
  - Childhood absence epilepsy
  - Juvenile absence epilepsy
  - Juvenile myoclonic epilepsy
  - Epilepsy with grand mal on awakening
  - Others
  - Seizures precipitated by specific modes of activation
- 2.2 Cryptogenic or symptomatic
  - West syndrome
  - Lennox-Gastaut syndrome
  - Epilepsy with myoclonic-astatic seizures
  - Epilepsy with myoclonic absences
- 2.3 Symptomatic
  - Early myoclonic encephalopathy
  - Early infantile epileptic encephalopathy with burst suppression
  - Others
  - Specific syndromes
  - Epilepsies in other disease states

3 **Undetermined whether focal or generalised**

4 **Special syndromes**
- 4.1 Febrile convulsions
- 4.2 Isolated seizures or isolated status epilepticus
- 4.3 Acute metabolic or toxic factors

*Adapted from Commission, 1989

Shortcomings of the present classification are well recognised even when it was first proposed (Commission, 1989). Patients may move from one syndrome to another during the evolution of the disorder. Some syndromes can be the result of many diseases and do not represent a single condition, e.g. Lennox-Gastaut syndrome, while the same pathology may manifest as different syndromes, e.g. tuberous sclerosis. The dichotomy between generalised and localisation-related (focal) is arbitrary and observational and heavily reliant upon EEG findings. However, bilateral EEG epileptiform discharges often have distinct regional accentuation or in fact, on closer inspection a focal origin with rapid secondary spread. The inadequacy of the present system is perhaps best illustrated by MTS, the most common pathological finding in refractory epilepsy (Semah et al, 1998). Whether it is the cause or consequence of mesial temporal lobe epilepsy (MTLE) and how it should be classified is one of the most fervent controversies in epileptology (Wolf, 1997). Advances in genetics have led to discovery of mutations of ion channels in some hereditary epilepsies (Steinlein et al, 1995; 1997; Singh et al, 1998; Wallace et al, 1998), which may influence the patient's response to drug treatment (Picard et al, 1999). It has also been shown that the same genetic defect can produce heterogeneous clinical phenotypes, which would be separated into different categories under the current system (Scheffer and Berkovic, 1997). An increasing number of structural abnormalities can be identified *in vivo* with more sophisticated neuroimaging, which may impart a different prognosis although all fall under the category of "focal symptomatic" epilepsies (Semah et al, 1998). The present classification systems are under current review to take into account underlying pathology more accurately to better reflect prognosis and other dimensions (Engel, 1998a).

1.4 Common aetiologies in adolescents and adults

The range of aetiologies of epilepsy varies in different age groups and geographical locations. Aetiologies in relation to age commonly encountered in industrialised countries

are shown in Figure 1. Generally speaking, epilepsy in childhood is most commonly caused by congenital and genetic conditions, which may only begin to manifest in late childhood or adolescence. In young adults, MTS, cortical dysgenesis, alcohol/drug abuse and trauma are important causes. In the elderly, cerebrovascular disease is common. Tumours and intracranial infections occur at all ages, although malignant neoplasm is more likely over the age of 30 years.

1.4.1 Idiopathic syndromes

Idiopathic generalised epilepsy (IGE) is a genetic disorder and the core group with onset in childhood or adolescence is currently subclassified into four distinct but overlapping syndromes (Table 2; Janz, 1997). It is debatable, however, whether they actually represent a biological continuum (Berkovic et al, 1987). They are characterised by a triad of seizure types – generalised tonic-clonic seizure (GTCS), generalised absence and myoclonus – with varying degree of dominance. Other shared features include a positive family history, onset in childhood or adolescence, 3 Hz spike and wave ictal EEG discharges that are often activated by hyperventilation or photic stimulation, and a good response to AED treatment (Duchowny and Harvey, 1996).

Juvenile myoclonic epilepsy is the most common idiopathic epilepsy syndrome, accounting for approximately 7% of all epilepsies (Dreifuss, 1989). Symptoms usually begin in the teenage years. The cardinal feature of JME is myoclonic seizures, which typically occur within the first few hours after waking, with very short and bilaterally symmetrical jerks that usually involve the upper extremities without impairing

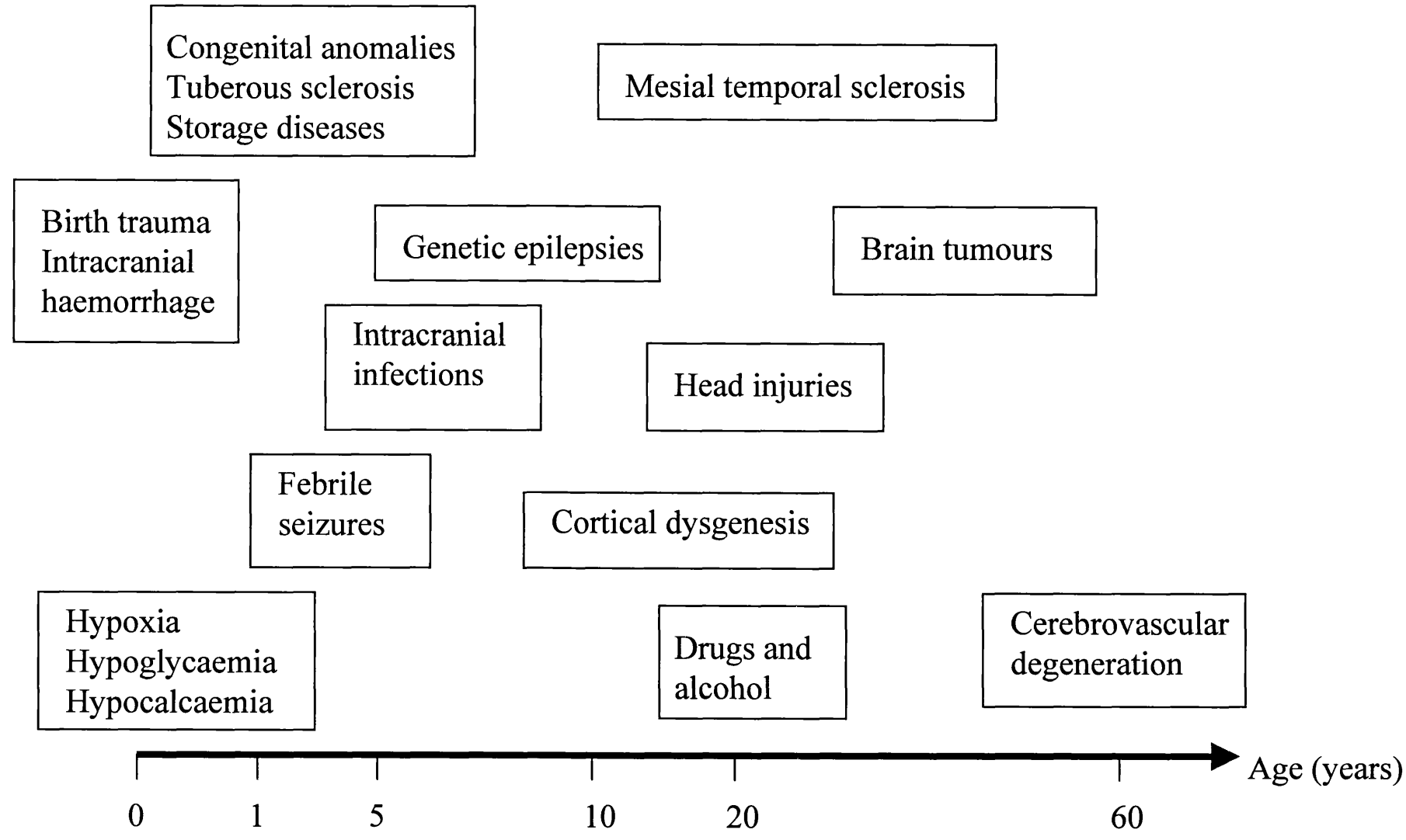


Figure 1. Aetiologies of epilepsy at different ages.

consciousness. They are exacerbated by sleep deprivation and alcohol intake and the patient may spill or drop things as a result. Myoclonic jerks may occur in series leading to GTCS, which occur in 90% of patients. About 30% of patients also complain of absence seizures. The condition is likely to be under-reported since the myoclonic jerks can be mild and easily overlooked. Linkage to chromosomes 6 (Greenberg et al, 1988) and 15 (Elmslie et al, 1997) has not been consistently demonstrated (Whitehouse et al, 1993; Elmslie et al, 1996; Obach et al, 2000) and polygenic inheritance is likely. The EEG shows a characteristic 3.5-6 Hz spike-and-wave pattern and multiple spike-and-wave complexes. The seizures respond well to treatment, but usually relapse when medication is withdrawn (Dreifuss, 1989).

Although childhood absence epilepsy has onset between 4 and 8 years of age, it may be undiagnosed until late childhood/adolescence. Absence seizures may be accompanied by eye deviation and retropulsion of the head and trunk, and can be very frequent, up to hundreds per day. Ictal EEG shows generalised symmetrical 3 Hz spike and wave discharges on a normal background. GTCS develop in 30-40% cases (Stefan and Snead, 1997).

The age of onset of juvenile absence epilepsy is around puberty. The absence seizures are less frequent than in childhood absence epilepsy, and retropulsive movements less common. There is more frequent association with GTCS and myoclonic seizures (Porter, 1993).

While GTCS occur in a great variety of epilepsy syndromes, epilepsy with grand mal on awakening refers to a specific syndrome with onset mostly in the second decade of life. GTCS occur exclusively, or predominantly, shortly after awakening and are exacerbated

by sleep deprivation and alcohol. There may be a second seizure peak in the evening period of relaxation. Absence and myoclonus are rare. Patients with GTCS without such diurnal pattern but fulfilling the criteria for a diagnosis of IGE, are classified as having IGE with GTCS (Janz and Wolf, 1997).

The other IGE syndromes (benign neonatal familial convulsions, benign neonatal convulsions and benign myoclonic epilepsy in infancy) and the idiopathic localisation-related epilepsies have onset in infancy or early childhood and are rarely seen in newly diagnosed patients at adult epilepsy centres.

There have been dramatic advances in the understanding of the genetic basis of the idiopathic epilepsies in the past decade (Treiman, 1993; Callenbach and Brouwer, 1997). The underlying genetic mutations in a number of animal models have been identified (Prasad et al, 1999) and single gene defects in ion channels (channelopathies) have been discovered in several, albeit relatively rare, epilepsy syndromes (Berkovic and Scheffer, 1997; Singh et al, 1998; Wallace et al, 1998). For the majority of common idiopathic syndromes (e.g. JME) multiple genes are likely to be involved with complex gene-environment interactions (Ottman, 1997; Serratosa, 1999).

1.4.2 Mesial temporal sclerosis

Mesial temporal sclerosis (MTS) is the most common pathology in intractable temporal lobe epilepsy, accounting for 50-70% of cases (Margerison and Corsellis, 1966; Falconer, 1974; Blümcke et al, 1999). The term "Ammon's horn sclerosis" was originally coined to describe the characteristic selective neuronal loss and gliosis involving the hippocampus proper (Sommer, 1880; Blümcke et al, 1999). MTS is used when other mesial temporal

structures such as the amygdala, uncus and parahippocampal gyrus are involved, as is often the case (Falconer and Taylor, 1968).

There is growing evidence to support the current concept that MTLE associated with MTS is a discrete syndrome with characteristic clinical features, although it is not recognised as such in the current ILAE classification (Engel, 1996a). Onset of seizures is usually before puberty, often preceded by febrile convulsions (Falconer et al, 1964; Annegers et al, 1987) or other initial injury, such as intracranial infection or head trauma, within the first 4 or 5 years of life (Mathern et al, 1995). There is an increased incidence of family history of seizure disorder (Engel, 1996a). In terms of semiology, a seizure typically begins with vegetative auras, such as epigastric rising, or affective symptoms (most commonly fear), but may consist of complex delusional experiences or hallucinations or olfactory or gustatory sensations, all indicative of involvement of mesial temporal limbic structures (Engel et al, 1997). Classically, the partial seizure remains unilateral and "simple", reflecting the fact that there is little transfer across the hippocampal commissure (Engel, 1996a). When complex partial seizure does ensue, impairment of consciousness is usually heralded by arrest and stare, followed by oroalimentary, gestural, and reactive automatisms lasting 1 to 2 minutes for which the patient is amnesic. Afterwards, the patient is confused for varying periods. There may be postictal dysphasia if the seizure involves the language-dominant hemisphere. Secondary generalisation is not uncommon.

The diagnosis of MTLE associated with MTS is supported by anterior temporal interictal spikes on a surface EEG, a characteristic unilateral sphenoidal ictal EEG onset pattern (Engel, 1996a), and hippocampal atrophy on T_1-weighted and increased mesial temporal signal on T_2-weighted magnetic resonance imaging (MRI; Bronen, 1992). Localised temporal lobe hypometabolism on positron emission tomography (PET) with

fluorodeoxyglucose, characteristic findings on magnetic resonance spectroscopy (MRS) and ictal or postictal single photon emission computerised tomography (SPECT), usually performed during pre-surgical evaluation, further support the diagnosis (Engel, 1996a).

Documentation of the natural history of MTS-related epilepsy is difficult due to its low prevalence (Murakami et al, 1996; van Paesschen et al, 1997a). Data derived from surgical series (Wieser, 1996; Briellman et al, 1998; Fisher and Blum, 1999) and specialist centres (Semah et al, 1998; Kim et al, 1999) suggest that patients with MTS have worse seizure control than those with other underlying abnormalities. However, 80% of patients can be rendered seizure-free by anteromesial temporal lobectomy, which may be performed without invasive evaluation in patients with refractory seizures who have a concordance of the above investigation findings all pointing to the same temporal lobe (Engel, 1996b).

Since its relationship with epilepsy was recognised nearly 200 years ago (Bouchet and Cazauvieilh, 1825), MTS has been the most intensively studied pathological abnormality associated with epilepsy, and its aetiology the most hotly debated. Whether it is the cause or consequence of seizures is highly controversial. That it is a result of damage by febrile seizures or other insults in an age-specific time window has long been postulated (Falconer et al, 1964; Mathern et al, 1995). This concept is supported by long-term prospective studies which confirm the association of prolonged febrile seizures and MTLE (Annegers et al, 1987), and recent documentation of evolution of MTS after prolonged febrile seizures and other acute insults in case series (Berkovic and Jackson, 2000). Animal data also show that chemically induced limbic seizures can result in histological changes resembling human MTS (Pringle et al, 1993; Liu et al, 1995). Other researchers point to the lack of association between the extent of MTS and seizure duration (Liu et al, 1995) and the observation that many patients with MTS lack a history of febrile seizures and that MTS is

often unilateral (Swanson, 1995). It is possible that both camps are correct: MTS is both the cause and effect of seizures, in patients who have pre-existing factors that predispose the hippocampus to injury (Fisher et al, 1998; Lewis, 1999; Berkovic and Jackson, 2000). Such factors may be developmental, such as cortical dysgenesis (Raymond et al, 1994; Lewis, 1999), or genetic (Kanemoto et al, 2000). Thus, prolonged seizures in predisposed individuals induce neuronal loss which, in turn, leads to formation of new synaptic connections (Pringle et al, 1993) that can express abnormal hyperexcitability and cause more seizures (Swanson, 1995; Blümcke et al, 1999), resulting in a vicious cycle that ultimately leads to refractory epilepsy. However, it remains unclear why MTS should be particularly pharmacoresistant compared to other pathologies. This will be discussed further in Part I; Section 5.

1.4.3 Other symptomatic causes

Cortical dysplasia (CD) is a heterogenous disorder of cortical development and organisation. It may be completely asymptomatic, but is often manifest as epilepsy. There may be associated learning disabilities or cognitive deficits, and systemic features may be present (e.g. cutaneous lesions in tuberous sclerosis). Previously only detected at autopsy, CD is now increasingly recognised as a significant cause of epilepsy owing to the advent of MRI (Shorvon, 1997). In one MRI study, 25% of patients with refractory, apparently cryptogenic epilepsy were found to have underlying CD (Raymond et al, 1995), suggesting it may also be particularly pharmacoresistant. CD may result from defects at any phase of neural development, and the pathological features depend largely on the timing of the defect in the developmental process and to a lesser extent on the cause of the defect. Based on MRI findings, a wide variety of changes are recognised, including abnormalities of neocortical gyration (e.g. lissencephaly), megalencephaly, heterotopias, tuberous sclerosis, focal cortical dysplasia, CD associated with neoplasia (e.g. dysembryoplastic

neuroepithelial tumour), dentate gyral abnormality, and microdysgenesis (Shorvon, 1997). The last abnormality has been suggested to underlie many "cryptogenic" epilepies or even IGE, but sophisticated MRI techniques are required for its detection *in vivo* (Sisodya et al, 1996).

Epilepsy is one of the most common symptoms of primary brain tumours (Lieu and Howng, 2000). Although they account for only approximately 5% of all newly diagnosed epilepsy (Hauser et al, 1993), 40% of adults presenting with new onset focal epilepsy are said to have cerebral neoplasms (Nashef, 1996). Slow growing tumours are more likely to present with seizures than rapidly growing ones (Villemure and de Tribolet, 1996). Ten to 30% of patients with chronic refractory focal epilepsy may have MRI-detectable low-grade cerebral tumours, many of which are missed by CT scan (Morris et al, 1998).

Vascular malformations include arteriovenous malformations (AVMs), cavernous haemangiomas, and venous and capillary malformations, although the latter is frequently an incidental finding and its causal relationship with epilepsy is unclear (Kraemer and Awad, 1994). AVMs are more likely to bleed (1-3% per year) than cavernous haemangiomas (around 0.5% per year; Moran et al, 1999). The latter is thought to be inherited in an autosomal dominant fashion with incomplete penetrance, and up to 75% of sporadic cases with multiple lesions are in fact familial cases when family members are screened by MRI (Labauge et al, 1998).

Stroke is the single most common cause of epilepsy in elderly people (Stephen and Brodie, 2000). The risk of single or recurrent seizures after a stroke is estimated to be around 10% in the first 5 years (Burn et al, 1997). Seizures are more likely to be associated with larger

haemorrhagic areas or infarction, and with cortical rather than subcortical involvement (Stephen and Brodie, 2000).

Post-traumatic epilepsy is an uncommon cause of epilepsy as a whole, but is considerably more frequent in the presence of depressed fracture, dural tear, focal signs, long post-traumatic amnesia and early seizures occurring within 1 week of the head injury (Jennett, 1975). Convulsions occurring immediately on impact do not carry an excess risk of late epilepsy (McCrory et al, 1997). There is no evidence that prophylactic AED treatment prevents the development of late post-traumatic epilepsy (Chadwick, 2000). Although seizures are frequent during the acute phase of bacterial or viral meningoencephalitis, CNS infections are uncommon causes of chronic epilepsy (Hauser et al, 1993).

2 Diagnosis of epilepsy

The diagnostic procedure aims to determine whether the patient has epilepsy, and if so, the syndromic classification and underlying aetiology which can have implications on prognosis and choice of therapy (Brodie and French, 2000). A wide range of conditions may mimic epileptic seizures and must be considered in the differential diagnosis. Syncopal attacks, during which there may be clonic movements and incontinence, are commonly misdiagnosed as epileptic seizures (Smith et al, 1999). Pseudoseizures or nonepileptic psychogenic seizures are estimated to account for 10 to 45% of patients with apparently refractory epilepsy (Devinsky, 1999). Misidentification of other conditions as epilepsy leads to unnecessary and potentially harmful treatments and delay in initiating appropriate therapy as well as other deleterious psychosocial and socioeconomic consequences (Smith et al, 1999). The temptation to attach a label of "epilepsy" should be resisted when there is doubt and the physician (and patient) should simply await the passage of time before coming to a firm conclusion.

2.1 History and examination

Despite dramatic advances in investigational techniques, the diagnosis of epilepsy remains essentially clinical, and is based on a detailed description of events experienced by the patient before, during, and after a seizure and, more importantly, on an eye witness account (Chadwick, 1994). Physical examination is usually unremarkable, although there may be focal neurological signs corresponding to any underlying focal structural abnormality.

2.2 Investigations

2.2.1 Neuroimaging

Neuroimaging aims to identify any underlying epileptogenic structural abnormalities and is indicated in patients suspected to have localisation-related epilepsy. MRI is undoubtedly the investigation of choice (Duncan, 1997). Since its initial application in 1984, its development has revolutionised the investigation and treatment of epilepsy. The superiority of MRI over X-ray computed tomography (CT) scanning in terms of sensitivity and specificity for identifying the aetiology of epilepsy in both adults and children is firmly established (Bronen, 1992; Duncan, 1997). The most common abnormalities identified by MRI are HS, CD, vascular malformations, tumours and acquired cortical damage. The "standard" MRI protocol includes T_1- and T_2-weighted sequences covering the whole brain in at least two orthogonal planes, with the minimum slice thickness possible on the scanner used. Although quantitative volumetric measurement of the hippocampus is often used in specialist centres and research, in clinical practice, visual inspection has been shown to be comparable in sensitivity and specificity in detecting HS (Bronen et al, 1997; Cheon et al, 1998; Kim et al, 1999). Rapid advances in MRI techniques, e.g. fluid attenuated inversion recovery (FLAIR) sequence, diffusion-weighted imaging, are likely to uncover the

underlying pathologies in many patients currently categorised as having "cryptogenic" epilepsy (Duncan, 1997).

CT should be used in preference to MRI only if the latter is contraindicated (e.g. in a patient with cardiac pacemaker, metal aneurysm clip, or severe claustrophobia), when there is the possibility of acute intracranial haematomas, or from a practical point of view, when MRI is not available.

Positron emission tomography (PET) may be used to map cerebral blood flow and regional cerebral glucose metabolism using appropriate radiolabelled tracers, and to demonstrate the binding of specific ligands. Single photon emission computerised tomography (SPECT) is less expensive than PET and has the added advantage of being able to detect changes in cerebral blood flow during a seizure, providing useful localisation information in partial seizures. Magnetic resonance spectroscopy (MRS) is another new technique for measuring cerebral metabolism, usually of selected neurotransmitters (Duncan, 1997). Currently, these imaging techniques are largely confined to presurgical evaluation or for research purposes.

2.2.2 Electroencephalography

Electroencephalography (EEG) remains valuable in providing support for the diagnosis of epilepsy and in aiding the classification of epilepsies and epilepsy syndromes (King et al, 1998). However, its limitations in the diagnostic setting must be recognised for proper interpretation (Binnie and Stefan, 1999). Approximately 0.5% of normal adults exhibit epileptiform activity on an EEG (Gregory et al, 1993). Even ictal EEG can be normal in partial seizures. About 15% of patients never show epileptiform activities on routine interictal EEG. A single routine interictal scalp EEG detects epileptiform abnormalities in

only 50% patients with epilepsy, although the sensitivity can be substantially increased by performing within 24 hours of a seizure (King et al, 1998), repeat recordings (Salinsky et al, 1987), or recording during sleep (Binnie and Stefan, 1999). Activation techniques, such as hyperventilation and photic stimulation, can be helpful in uncovering abnormalities and identifying precipitating factors. Video-EEG telemetry is an essential part of preoperative evaluation for epilepsy surgery, and can provide conclusive evidence in diagnosing non-epileptic pseudoseizures.

3 Treatment of epilepsy

3.1 Historical perspective

> "There is scarcely a substance in the world capable of passing through the gullet of man that has not at one time or other enjoyed the reputation of being an antiepileptic." – Sir Edward Henry Sieveking, 1861.

Even when Hippocrates was trying to rationalise the condition, a regime of drugs, lifestyle changes, and dietary manipulation was formulated to treat epilepsy (Temkin, 1971). Dietary measures ranged from the abstention from meat to the absolute avoidance of all food and drink. Phlebotomy and the drinking of urine or vinegar were other simple regimens, while more peculiar procedures included the provocation of sneezing before going to bed, rubbing of affected limbs with the genitals of the seal, or the ingestion of the genitals of various exotic animals. In many different cultures, skull trephination was carried out in an attempt to allow the escape of evil spirits (Gross, 1992).

Confusion between epilepsy, madness and spiritual possession in the Middle Ages might explain the continued reliance on magical potions or religious rituals, even by physicians. Although Jackson's observations in the late 19th century with regard to the physiological

basis of epilepsy were surprisingly accurate for their time, he continued to claim that " there can be no question that ligature is a most valuable means of arresting such fits" (Jackson, 1873). Silver nitrate was commonly used in hospices around England, and Gowers listed mistletoe, turpentine, trephining and castration or circumcision as possible treatments (Gross, 1992).

Amid such a plethora of unproven (and ineffective) therapeutic interventions, bromide was welcomed enthusiastically as the first pharmacological treatment with demonstrated benefits. Working on the erroneous assumption that epilepsy was a manifestation of either hysteria or sexual frustration, Sir Charles Locock was able to achieve unprecedented results with potassium bromide (Locock, 1857). An improvement among 84% patients and a seizure-free rate of 32% were enviable even by today's standard (Hammond, 1871). The ascendant position of bromides was further confirmed by Gowers, who might also be credited to be the first to advocate adjunctive pharmacotherapy including digitalis, belladona and cannabis (Gowers, 1881). By the end of the 19th century, 4 kg of bromides were being used annually at the National Hospital for Nervous Diseases (Shorvon and Sander, 1996). However, the toxicity of bromide therapy was soon to be recognised, notably afflictions of the skin and mental slowing, rendering it obsolete once a better tolerated alternative was found.

The discovery of the anticonvulsant properties of phenobarbital (PB) in 1912 heralded the beginning of the modern pharmacotherapy of epilepsy (Table 3). Unable to get a night of uninterrupted sleep having been assigned to a room above his ward of patients with epilepsy, Alfred Hauptmann, a German physician, provided them with a blanket prescription of PB as a hypnotic. To his surprise and delight, seizure frequency was reduced in many patients (Brodie and Dichter, 1997). In spite of its propensity to cause

Table 3. Currently available antiepileptic drugs (AEDs) and the UK launch dates of the new agents.

"Established" AEDs	"New" AEDs	
Barbiturates (phenobarbital, primidone)	Vigabatrin*‡	1989
Benzodiazepines (clobazam, clonazepam)	Lamotrigine	1991
Phenytoin	Gabapentin*†	1993
Carbamazepine	Topiramate*	1995
Sodium valproate	Tiagabine*	1998
Ethosuximide	Fosphenytoin	1998
Acetazolamide	Oxcarbazepine	2000
	Levetiracetam*	2000
	Felbamate†	) Available in
	Zonisamide	) other countries

* Only licensed as add-on treatment in the UK.

† Licensed as monotherapy in some countries but not in the UK.

‡ Licensed as monotherapy for infantile spasms.

sedation and other cognitive and behavioural side effects, PB remained unrivalled for the treatment of epilepsy for almost 30 years and is still widely used in developing countries as it is inexpensive and easy to use. Phenytoin (PHT) was discovered as the result of the search by Merrit and Putnam (1938) for a non-sedatory analogue of PB by screening a wide variety of compounds against electrically induced seizures in animals. Carbamazepine (CBZ) was manufactured by Schindler at Geigy in 1953. It was originally developed as an antipsychotic and its antiepileptic properties were not observed clinically until 1963 (Brodie and Dichter, 1997). The clinical utility of sodium valproate (VPA) was unknown until its anticonvulsant activity was fortuitously discovered in 1963 by Meunier who used it as a solvent in testing other potential AEDs (Fariello et al, 1995). Ethosuximide (ESM) for absence seizures, primidone, a prodrug of PB, acetazolamide, and some benzodiazepines (BDZ), particularly clobazam (CLB) and clonazepam, have also become available between the 1950s and 1980s. But it was the late 1980s when the number of AEDs started to increase dramatically (Figure 2).

No less than 9 new AEDs have become available worldwide since 1989. Lamotrigine (LTG) was developed on the erroneous hypothesis that the effectiveness of an AED might be related to its antifolate properties (Reynolds et al, 1966). Zonisamide (ZNS), felbamate (FBM) and topiramate (TPM) were discovered by random drug screening using animal seizure models (White, 1997). Oxcarbazepine (OXC) was derived from CBZ in order to mimic the latter's action while improving its side effect profile (Macdonald and Kelly, 1995). Fosphenytoin is a prodrug of PHT and was developed as an alternative method of administering parental PHT in status epilepticus with better tolerability and safety profile (Luer, 1998). Vigabatrin (VGB), tiagabine (TGB) and gabapentin (GBP) can be claimed as the results of rational drug development. They were designed to facilitate the inhibitory action of γ-aminobutyric acid (GABA), although the mechanism of GBP remains to be

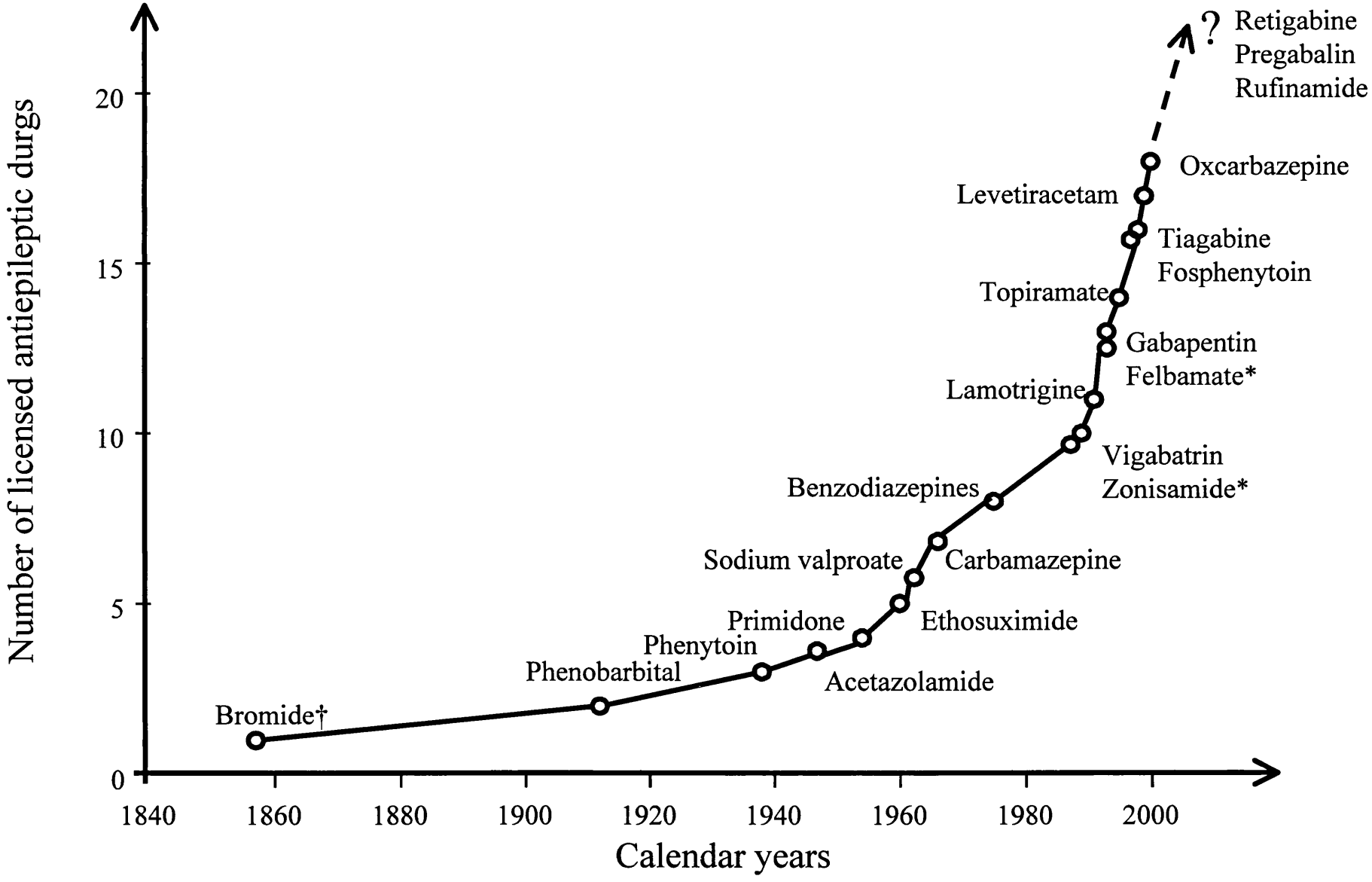


Figure 2. Progress in antiepileptic drug development in the UK and worldwide. († disused, * currently not available in the UK)

definitively determined. The anticonvulsant effect of levetiracetam (LEV), the latest AED introduced, was initially undetected by the traditional seizure models and only discovered by further investigation in alternative models (Bialer et al, 1999). Retigabine, pregabalin, and rufinamide, to name only a few, are compounds currently under development as novel AEDs.

Such rapid expansion of the pharmacopoeia in the last decade results partly from greater appreciation of the role of amino acids in neurotransmission, leading to a search for drugs with discrete, well-defined neurochemical actions. It is also testimony to the fact that many patients continue to suffer from recurrent seizures despite AED treatment. The "magic bullet" to pass through their gullet is yet to be found.

3.2 Mechanisms of action of antiepileptic drugs

It is important to appreciate the mode of action of the AEDs since it may be one of the criteria when making an appropriate drug choice for the individual patient, both as monotherapy and in combination treatment. Patients not responding to one AED may become seizure-free when changed to another with a different mode of action (Brodie et al, 1999b). A mechanistic approach may also provide a template for rational combination therapy. There is accumulating clinical data suggesting combination of a Na^+ channel blocker with a drug that enhances GABAergic inhibition or has multiple actions may be efficacious (Stolarek et al, 1994; Brodie et al, 1997b; Deckers et al, 2000). In addition, understanding the mode of action of the AEDs may shed light on the pathophysiology of seizures and epilepsy, which in turn may provide a framework for more rational drug development.

Although the mode of action of the currently marketed AEDs is still not fully understood, at the cellular level, three basic mechanisms are recognised (Table 4): modulation of voltage-dependent ion (Na^+, Ca^{2+}, K^+) channels, enhancement of GABA-mediated inhibitory neurotransmission, and inhibition of excitatory (particularly glutamate-mediated) transmission.

These targets of AED action will be briefly discussed and the mechanisms of action of the AEDs relevant to the studies described in this thesis will be outlined below. They include the established drugs PB, PHT, CBZ and VPA, and the newer agents LTG, OXC, GBP, TPM, VGB and TGB. They are grouped below according to their "primary" mode of action, although many less well-characterised, "minor" mechanisms may well contribute to a drug's antiepileptic effect. Some drugs exert their antiepileptic effect via multiple mechanisms, while the primary mode of action of some others is yet to be discovered.

3.2.1 Sodium channels

In the nervous system, voltage-gated ion channels control the flow of cations across surface and internal cell membranes (Barchi, 1998). Of these, the Na^+ channel is arguably of principal importance. The main structural component of the neuronal Na^+ channel is the α-subunit which forms the ion conducting pore and confers voltage-dependency (Catterall, 1992). In mammalian brain, the α-subunit associates with two auxiliary subunits designated β1 and β2. The β-subunits are not required for basic Na^+ channel activity, but modulate the expression and function of individual channels (Ragsdale and Avoli, 1998).

Voltage-dependent Na^+ channels are responsible for the upstroke of the neuronal action potential and ultimately control the intrinsic excitability of the nervous system (Porter and

Table 4. Proposed mechanisms of action of antiepileptic drugs. (Upton, 1994; Macdonald and Kelly, 1995; Schachter, 1995; Meldrum, 1996; Coulter, 1997; White, 1999)

	$\downarrow Na^+$ channels	$\downarrow Ca^{2+}$ channels	$\uparrow K^+$ channels	$\uparrow$ GABA transmission	$\downarrow$ glutamate transmission
Established					
Phenytoin	+++				
Carbamazepine	+++				+
Valproate	+	+		++	+
Ethosuximide		+++			
Phenobarbital		+		+++	+
Benzodiazepines				+++	
Newer					
Lamotrigine	+++	+			
Vigabatrin				+++	
Gabapentin	+	+		++	
Topiramate	++	++		++	++
Felbamate	++	+		++	++
Tiagabine				+++	
Oxcarbazepine	+++	+	+		
Zonisamide	++	++			
Levetiracetam		+		+	+

+++ = primary action; ++ = probable action; + = possible action

Rogawski, 1992). At normal membrane potentials, most Na^+ channels exist in a closed, resting state. Upon depolarisation, the channel activates, facilitating ion flux. Thereafter, the Na^+ channel enters an inactivated state from which it is not readily re-activated. Repolarisation of the neuronal membrane rapidly converts the channel back to a resting state, from which it can respond to subsequent depolarisations (Catterall, 1992; Ragsdale and Avoli, 1998). Neuronal Na^+ channels can cycle through these functional states within a few milliseconds. This characteristic is essential for sustaining the rapid bursts of action potentials necessary for some normal brain functions and is implicated in epileptic discharges. The neuronal Na^+ channel represents one of the most important targets for AED action (Upton, 1994; Macdonald and Kelly, 1995; Meldrum, 1996; White, 1999).

3.2.2 Calcium channels

Voltage-sensitive Ca^{2+} channels can be broadly classified into low or high threshold, according to the membrane potential at which they are activated (Hofmann et al, 1994). The low threshold T-type Ca^{2+} channel is expressed predominantly in thalamocortical relay neurones where it is believed to be instrumental in the generation of the rhythmic 3-Hz spike-and-wave discharge, characteristic of generalised absence seizures (Coulter et al, 1989a). It is the well-recognised target of ESM (Coulter et al, 1989b; 1989c). High threshold Ca^{2+} channels are subclassified by their pharmacological properties into L-, N-, P-, Q- and R-types (Hofmann et al, 1994; Catterall, 1995; Dolphin, 1995). These channels are distributed throughout the nervous system on dendrites, cell bodies and nerve terminals. The N-, P- and Q-type channels, in particular, have been implicated in the control of neurotransmitter release at the synapse (Stefani et al, 1997).

Interest in Ca^{2+} channels has heightened in recent years following the identification of subunit specific genetic mutations which can alter channel structure and/or function and

which have been implicated in several human neurological diseases (Ophoff et al, 1998). Several AEDs have also been reported to block voltage-sensitive Ca^{2+} channels, in a subtype-specific manner, an effect that may contribute to their antiepileptic actions (Stefani et al, 1997).

3.2.3 Potassium channels

At the neuronal level, K^+ channels are intimately involved in excitability. They are responsible for the action potential downstroke or, more specifically, repolarisation of the plasma membrane in the aftermath of Na^+ channel activation (Pongs, 1999). Direct activation of voltage-dependent K^+ channels hyperpolarises the neuronal membrane and limits action potential firing (Porter and Rogawski, 1992). Accordingly, K^+ channel activators have anticonvulsant effects in some experimental seizure models (Gandolfo et al, 1989; Rostock et al, 1996), whereas K^+ channel blockers precipitate seizures (Yamaguchi and Rogawski, 1992).

3.2.4 GABA-mediated inhibition

GABA is the predominant inhibitory neurotransmitter in the mammalian central nervous system where it is released at up to 40% of all synapses (Olsen and Avoli, 1997). GABA is synthesised from glutamate, exclusively in GABAergic neurones, by the action of the enzyme glutamic acid decarboxylase (GAD; Löscher, 1999). Upon synaptic release, GABA acts on its three specific receptors, $GABA_A$, $GABA_B$ and the newly characterised $GABA_C$. GABA receptors are distinguished by their pharmacology and functions (Johnston, 1996). The $GABA_A$ receptor belongs to the ligand-gated ion channel superfamily and responds to GABA binding by increasing Cl^- conductance, resulting in neuronal hyperpolarisation (Rabow et al, 1995). $GABA_B$ receptors are G-protein-linked, activation of which leads to an increase in K^+ conductance (Olsen and Avoli, 1997). It has

recently been proposed that $GABA_A$ and $GABA_B$ receptors may have evolved from the $GABA_C$ receptor which is comparatively simpler in structure and pharmacology (Johnston, 1996).

Following receptor activation, GABA is removed from the synaptic cleft, into localised nerve terminals and glial cells, by specific membrane-bound transport molecules. Currently, four active transport systems, GAT-1, GAT-2, GAT-3 and BGT-1, have been described (Borden et al, 1992). GABA has a variable affinity for these transporters and only GAT-1, predominantly located in the cerebral cortex and hippocampus, has GABA as its principal substrate (Gaustella et al, 1990). After removal from the synapse, GABA is either recycled to the readily releasable neurotransmitter pool (GABAergic nerve terminals only) or metabolised (neurones and glial cells) to the inactive molecule succinic acid semialdehyde by the action of the mitochondrial enzyme GABA-transaminase (GABA-T; Meldrum, 1995).

Impairment of GABA function is widely recognised to provoke seizures, whereas facilitation has an anticonvulsant effect (Löscher, 1999). Accordingly, potentiation of GABA is regarded as one of the most attractive, and successful, mechanisms of AED action and can be achieved by increased synthesis, increased release, allosteric receptor facilitation or reduced inactivation (Sills et al, 1999; Figure 3).

3.2.5 Glutamate-mediated excitation

Glutamate is the principal excitatory neurotransmitter in the mammalian brain (Meldrum, 2000). Focal injection of glutamate induces seizures in animals, and over-activation of glutamatergic transmission or abnormal glutamate receptor properties are observed in certain experimental seizure models and human epilepsy syndromes (Meldrum, 1995).

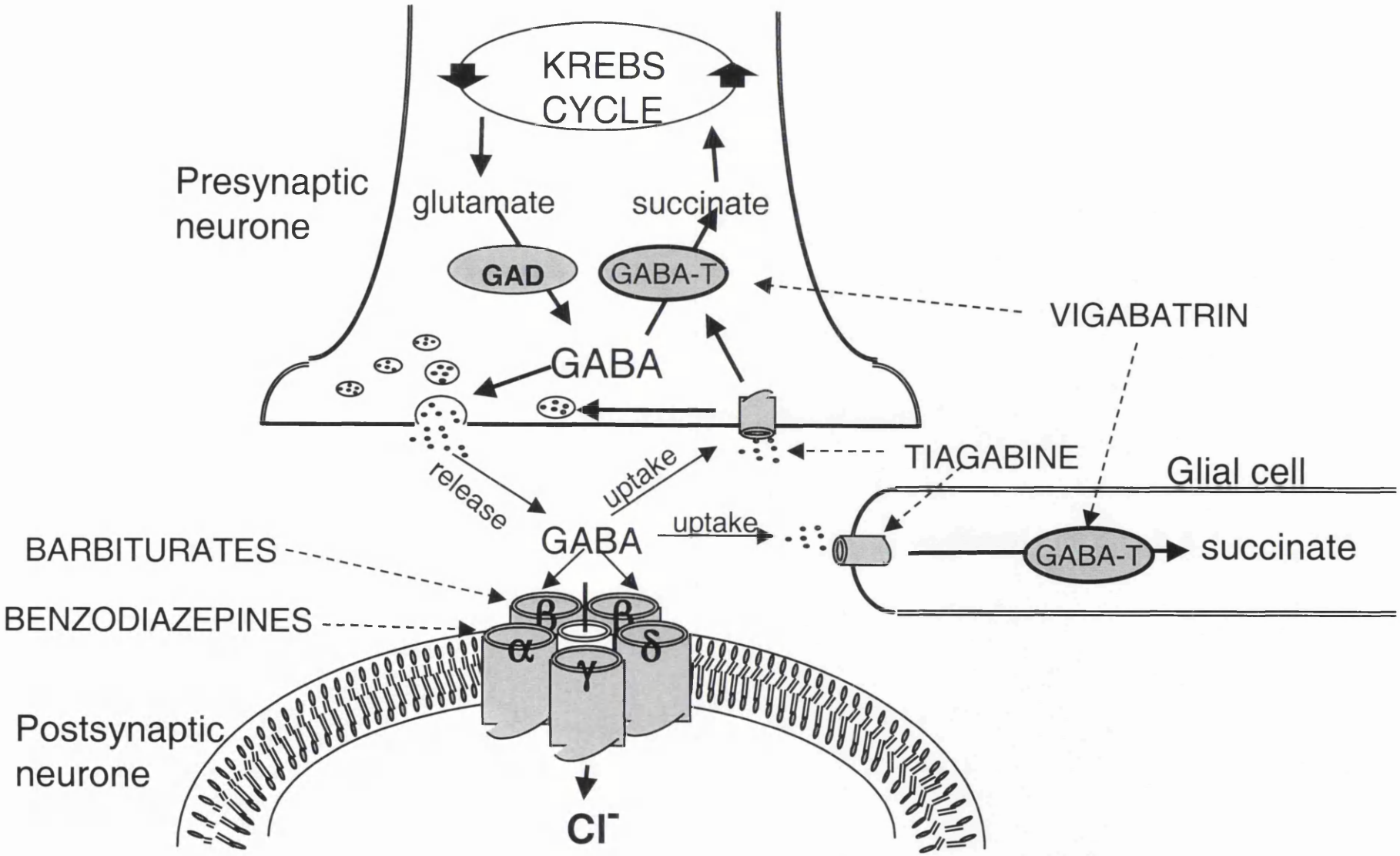


Figure 3. Metabolism and uptake of GABA and site of action of GABAergic antiepileptic drugs.
GABA=γ-aminobutyric acid, GABA-T=GABA-transaminase, GAD=glutamic acid decarboxylase.

Following synaptic release, glutamate exerts its pharmacological effects on several receptors, categorised into ionotropic and metabotropic families. The former are classified into three specific subtypes, AMPA (α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid), kainate and NMDA (N-methyl-D-aspartate), which form ligand-gated ion channels, permeable to Na^+ and, depending on subtype and subunit composition, Ca^{2+} ions (Trist, 2000). The NMDA receptor is further distinguished by having glycine as a co-agonist. The AMPA and kainate subtypes of glutamate receptor are implicated in fast excitatory neurotransmission, whereas the NMDA receptor, quiescent at resting membrane potential, is recruited during periods of prolonged depolarisation (Meldrum, 2000). The metabotropic family of glutamate receptors, also classified into three distinct subtypes (Groups I, II and III), are G-protein linked and predominantly pre-synaptic, possibly controlling neurotransmitter release (Meldrum, 2000).

Although none of the commonly used AEDs exert their pharmacological effects solely by an action on the glutamate system, blockade of ionotropic glutamate receptors is believed to contribute to the antiepileptic activity of several compounds (Upton, 1994; Macdonald and Kelly, 1995; Meldrum, 1996; White, 1999). In addition, several AEDs have been reported to reduce glutamate release, although this effect may be more indicative of their actions on neuronal calcium channels than a direct effect on the glutamate system (Stefani et al, 1997).

3.2.6 Modulation of ion channels

3.2.6.1 Phenytoin

PHT is the prototypic antiepileptic Na^+ channel blocker (Rogawski and Porter, 1990; Ragsdale and Avoli, 1998). It blocks Na^+ currents in a voltage- and frequency-dependent fashion by prolonging the inactivation phase (Tunnicliff, 1996). As a result, it produces a

reduction in the frequency of sustained repetitive firing of action potentials without affecting their amplitude or duration (McLean and Macdonald, 1983). An effect on persistent Na^+ currents by PHT has also been suggested (Lampl et al, 1998).

3.2.6.2 Carbamazepine

Like PHT, CBZ has been shown to stabilise the inactive form of Na^+ channels in a voltage-, frequency-, and time-dependent fashion (Courtney and Etter, 1983). However, subtle but important differences in binding characteristics (Kuo et al, 1997) and effectiveness of blockade (Schwartz and Grigat, 1989) between the two drugs may contribute to their different clinical profile (see Table 5). CBZ may also exert its antiepileptic effect via inhibition of glutamatergic transmission (Waldmeier et al, 1995; Hough et al, 1996). Effects on the serotonin (Dailey et al, 1997a; 1997b) and adenosine (Marangos et al, 1983) systems have been reported. Whether these additional actions contribute to the anticonvulsant effects of the drug is unclear.

3.2.6.3 Lamotrigine

There is no evidence that LTG's antiepileptic activity is related to its weak antifolate effect (Macdonald and Kelly, 1995). Instead, like PHT and CBZ, it acts primarily by blocking Na^+ channels in a voltage- and use-dependent fashion (Cheung et al, 1992; Lang et al, 1993; Zona and Avoli, 1997). Its broader clinical and preclinical profiles suggest LTG may differ from PHT and CBZ in its effects on Na^+ currents. Unlike PHT, LTG acts principally on the slow inactivated state of the sodium channel (Kuo and Lu, 1997). In addition, LTG may exhibit differential sensitivity for the various α-subunits of the channel (Coulter, 1997). It has been suggested that LTG may selectively target Na^+ channels on neurones that synthesise glutamate and aspartate (Leach et al, 1986)

In addition to Na^+ channel effects, LTG reduces whole-cell Ca^{2+} currents in rat amygdalar neurones, possibly via the N- and P-type channels (Wang et al, 1996; Stafani et al, 1997). This might explain the inhibition of electrically stimulated glutamate release from rat spinal dorsal horn slices observed with the drug (Teoh et al, 1995). It remains possible, however, that LTG possesses additional unidentified mechanisms that confer its relatively broader clinical spectrum when compared with other Na^+ channel blocking agents.

3.2.6.4 Oxcarbazepine

OXC is a structural analogue of CBZ. Keto substitutions at the 10, 11 positions of the dibenzazepine nucleus do not affect the therapeutic profile of the drug when compared to CBZ, but result in altered biotransformation and better tolerability (Macdonald and Kelly, 1995). OXC is essentially a pro-drug and is rapidly and completely reduced in the liver to its active metabolite, 10, 11-dihydro-10-hydroxycarbazepine (MHD; McLean et al, 1994). Both OXC and MHD appear to act primarily by blocking voltage-dependent Na^+ channels in a manner similar to that reported for PHT and CBZ (McLean et al, 1994). Blockade of high-threshold Ca^{2+} currents (Stefani et al, 1997) and promotion of K^+ channel conductance (McLean et al, 1994) have also been suggested.

3.2.7 Potentiation of GABA

3.2.7.1 Barbiturates and benzodiazepines

PB exerts its pharmacological effects by allosteric activation of the $GABA_A$ receptor, increasing the duration of chloride channel opening, without affecting frequency of opening or channel conductance (Macdonald et al, 1989; Twyman et al, 1989). The barbiturates can also activate the $GABA_A$ receptor directly, in the absence of GABA, an effect that may underlie their sedative properties (Rho et al, 1996; White, 1999).

Unlike PB, the benzodiazepines, such as clonazepam and clobazam, increase the frequency of chloride channel opening, without affecting open duration or channel conductance (Study and Barker, 1981; Twyman et al, 1989). They are unable to directly activate the $GABA_A$ receptor in the absence of GABA (White, 1999).

3.2.7.2 Vigabatrin

VGB is an ethyl analogue of GABA that is widely recognised to exert its pharmacological effects by irreversible inhibition of GABA-T, the enzyme responsible for the catabolism of GABA (Jung et al, 1977). As a consequence, it elevates GABA levels, and thus potentiates inhibitory neurotransmission, throughout the brain (Schechter et al, 1977). A single dose of VGB reduces mouse brain GABA-T activity to around 20% of control levels and produces a 4 – fold increase in whole brain GABA concentrations. This effect persists for over 24 hours, with GABA-T activity and GABA concentrations only returning to normal upon the synthesis of new enzyme protein over a period of 4 – 5 days (Schechter et al, 1977). Similar effects on the GABA system are also observed in man (Petroff et al, 1996a).

3.2.7.3 Tiagabine

TGB is an analogue of nipecotic acid, a prototypic GABA uptake blocker, which is widely recognised to prevent GABA transport into both neurones and glial cells (Krogsgaard-Larsen et al, 1987). Nipecotic acid, however, fails to cross the blood-brain barrier following systemic administration. This problem is overcome by linking it to a lipophilic anchor to form TGB which is able to cross the blood-brain barrier more readily (Brodie, 1995). TGB inhibits GABA uptake predominantly by an action on the GAT-1 transporter with little or no activity on GAT-2 or GAT-3 (Braestrup et al, 1990; Borden et al, 1994). As a result, the pharmacological effects of TGB reflect the regional distribution of GAT-1

and are mainly restricted to the cerebral cortex and hippocampus (Meldrum and Chapman, 1999).

Unlike VGB, which elevates whole brain GABA concentrations, TGB temporarily prolongs the presence of GABA in the synaptic cleft (Sills et al, 1999). In rat hippocampal slices, TGB prolongs the duration, but not the magnitude, of the peak inhibitory postsynaptic current, consistent with delayed clearance of GABA from the synapse (Roepstorff and Lambert, 1992). Inhibition of GABA uptake is the only known mechanism of TGB action.

3.2.8 Drugs with multiple mechanisms of action

3.2.8.1 Sodium valproate

The precise mechanisms by which VPA exerts its antiepileptic effects remain to be conclusively determined. One possible action is blockade of voltage-dependent Na^+ channels, in a manner similar to that reported for PHT and CBZ (McLean and Macdonald, 1986; van Dongen et al, 1986; Zona and Avoli, 1990). Another possible mechanism of action is inhibition of Ca^{2+} currents in a fashion similar to that described for ESM, which may explain its effectiveness against generalised absence seizures. However, its reduction of T-type Ca^{2+} currents in rat primary afferent neurones is modest and requires relatively high concentrations (Kelly et al, 1990). There is evidence to suggest that VPA elevates whole brain GABA levels and potentiates GABA responses, possibly by enhancing GAD activity and inhibiting GABA degradation (Löscher, 1999). Acute doses of VPA decrease brain levels of the excitatory amino acid aspartate, without influencing those of glutamate or GABA (Schechter et al, 1978). The potential contribution of any of the above effects to the clinical activity of VPA remains to be determined.

3.2.8.2 Topiramate

The sulfamate derivative TPM possesses multiple mechanisms of action, including inhibition of Na^+ and Ca^{2+} currents, blockade of the AMPA/kainate subtype of glutamate receptor, and facilitation of GABA. It also inhibits carbonic anhydrase, although this effect is not believed to contribute to its antiepileptic action (Shank et al, 1994).

TPM reduces voltage-dependent Na^+ currents in cultured cerebellar granule cells (Zona et al, 1996). In this respect, its action is similar to that of PHT and CBZ, delaying recovery of Na^+ channels from the inactive state. It has also been reported that TPM blocks whole cell Ca^{2+} currents, possibly by an action on the high-voltage-activated L-type channel (Zhang et al, 2000a). In hippocampal neurones, TPM blocks inward currents evoked by kainate but not NMDA (Coulter et al, 1995), implying a selective effect on the AMPA / kainate subtype of glutamate receptor. TPM also interacts with the $GABA_A$ receptor. It enhances GABA-stimulated chloride flux into cerebellar granule neurones and increases chloride currents evoked by GABA in mouse cerebral cortical neurones (White et al, 1997). TPM elevates brain levels of GABA and its metabolites in patients with refractory epilepsy (Petroff et al, 1999).

3.2.8.3 Gabapentin

GBP was originally designed to mimic the action of GABA, but subsequent studies have shown that it does not directly interact with GABA receptors (Taylor et al, 1998) or transporters (Su et al, 1995; Macdonald and Greenfield, 1997). There is evidence that GBP may increase the synthesis (Taylor et al, 1992) and non-vesicular release of GABA (Gotz et al, 1993) and prevent its metabolism (Leach et al, 1997). Using [^{1}H] nuclear magnetic resonance spectroscopy, GBP has been shown to elevate GABA concentrations in the occipital cortex of epileptic patients (Petroff et al, 1996b). Whether this observation

is the result of enhanced synthesis, increased release or reduced metabolism of GABA remains to be determined.

Early efforts to identify the mechanism of GBP proposed an interaction with the L-amino acid transport system resulting in alterations in the cytosolic and extracellular concentrations of several amino acids, including L-leucine, L-valine and L-phenylalanine (Su et al, 1995). Inhibition of branched chain amino acid transferase and augmentation of glutamate dehydrogenase activities have also been mooted (Goldlust et al, 1995). Evidence supporting an influence on neuronal firing via an effect on Na^+ channel function by GBP is conflicting (Taylor et al, 1988; Wamil and McLean, 1994).

The identification of a specific binding site for GBP in mammalian brain, and its subsequent unveiling as the $\alpha 2\delta$ subunit of the L-type voltage-dependent calcium channel (Gee et al, 1996), suggested another potential pharmacological mechanism. The implications of these findings remain to be fully investigated, but the lack of effect of GBP on whole cell Ca^{2+} currents in human dentate granule cells acutely isolated from patients with temporal lobe epilepsy (Schumacher et al, 1998) questions the relevance of this finding.

3.3 Clinical use of antiepileptic drugs

3.3.1 Initiation of treatment

The goal of treatment should be restoration of a normal life through complete control of seizures with minimal or no side effects, unless there is a good reason to be less ambitious. Most patients with recurrent seizures require treatment. The exceptions are those with provoked seizures which can be avoided by lifestyle changes (e.g. alcohol related seizures), or those with widely separated episodes (Brodie and Dichter, 1996). The question of

whether to treat a single seizure is contentious. Depending on other risk factors, only 30-70% of people will have further attacks (i.e. epilepsy) after an unprovoked seizure (Hauser et al, 1990; Shinnar et al, 1990). A recent multicentre randomised study showed that while treatment after a single seizure reduced the chance of recurrence, it did not alter the long-term prognosis (Musicco et al, 1997). All AEDs have side effects, some potentially serious. It would, therefore, seem reasonable to withhold treatment until a second event occurs. Patients with underlying cerebral lesions or specific syndromes, such as juvenile myoclonic epilepsy, are at higher risk of developing recurrent seizures and should probably be treated after one episode (Brodie and Dichter, 1997). The wishes of the patient, which may include consideration of employment and driving, should also be taken into account. The decision about treatment should only be made after ample discussions with the patient about the risks and benefits of treatment (Perucca et al, 2000). Treatment is doomed to failure in an uncooperative patient. Compliance can be monitored for some drugs with the aid of measurement of plasma concentration (McKee and Brodie, 1997).

Whether used as monotherapy in a newly diagnosed patient or polytherapy for refractory seizures, AEDs should be chosen by "matching" the individual characteristics of the patients (age, gender, body habitus, concomitant disease and medications), the seizure disorder (seizure type, epilepsy syndrome), and the drug (mode of action, range of efficacy, tolerability, side effect and interaction profiles; Tables 5 and 6).

3.3.2 Monotherapy for newly diagnosed epilepsy

Since the advantages of monotherapy over multiple-drug treatment, particularly in terms of side effect profile, were demonstrated by a series of studies in the late 1970s and early 1980s (Shorvon and Reynolds, 1977; Shorvon and Reynolds, 1979; Reynolds and Shorvon,

Table 5. Efficacy of antiepileptic drugs against common seizure types (Brodie and Kwan, 2001).

Drug	Partial	Secondary generalised	Tonic-clonic	Absence	Myoclonic
Carbamazepine	+	+	+	-	-
Gabapentin	+	+	?+	?	-
Lamotrigine	+	+	+	+	+
Oxcarbazepine	+	+	+	?	?
Phenobarbital	+	+	+	0	?+
Phenytoin	+	+	+	-	-
Sodium valproate	+	+	+	+	+
Tiagabine	+	+	?+	-	?
Topiramate	+	+	+	?	+
Vigabatrin	+	+	0	-	-

Key: + proven efficacy, ?+ probable efficacy, 0 ineffective, - worsens seizures, ? unknown

Table 6. Factors to be considered when choosing an antiepileptic drug regimen (Brodie and Kwan, 2001).

Patients	Seizures	Drugs
Infants/Children	First seizure	Mechanism
Teenagers	Newly diagnosed	Kinetics
Pregnancy	Idiopathic	Efficacy
Elderly	Localisation-related	Toxicity
Learning disabled	Syndrome	Interactions
Concurrent diseases	Refractory	Teratogenicity

1981; Schmidt, 1982; Schmidt, 1983), monotherapy has rightly become the established principle for managing new onset epilepsy. Significant differences in efficacy as monotherapy have not been consistently demonstrated for the common seizure types in clinical trials among the established AEDs (Mattson et al, 1985; Callaghan et al, 1985; Mattson et al, 1992; Richens et al, 1994; De Silva et al, 1996). A few studies have compared a modern agent with an established one (Brodie et al, 1995; Bill et al, 1997; Guerreiro et al, 1997; Christie et al, 1997; Chadwick et al, 1998; Brodie et al, 1999a, Chadwick, 1999) or with another new AED (Brodie et al, in press). Since the majority of newly diagnosed patients will have a good prognosis (see Part I; Section 4.2), the emphasis for them is tolerability and the avoidance of potentially serious side effects or chronic complications of long-term treatment (Brodie, 1999).

3.3.3 Combination therapy for refractory epilepsy

The controversy of management strategy arises mainly after the first AED proves ineffective. Despite being commonly used in patients unresponsive to monotherapy, when and how AED combinations should be employed has not been well studied. An alternative drug is unavoidable when the patient develops intolerable adverse events, but when seizures persist despite a sufficient dose being tolerated, it is unclear whether or not substitution should be tried before a combination of two drugs is used (Schmidt and Gram, 1995). Advocates of persistence with alternative monotherapy point to the toxicity of polytherapy and success of alternative drugs, as demonstrated in the Veteran Affairs study (Mattson et al, 1985). However, in this study many patients were initially treated with PB or primidone, neither of which would nowadays be considered as a first line drug in industrialised countries. The arrival of new AEDs in the past decade, with their diverge mechanisms of action and generally better tolerability, has raised the possibility of

effective and safe combinations for patients unresponsive to monotherapy (Brodie, in press).

Although data directly evaluating the effectiveness of AED combinations are scarce, some regimens, such as VPA with ESM for absence seizures (Rowan et al, 1983), VPA with LTG for partial-onset and generalised seizures (Brodie et al, 1997b), LTG with VGB for partial seizures (Stolarek et al, 1994; Schapel et al, 1996), VGB and TGB for partial seizures (Leach and Brodie, 1994), and LTG with TPM for a range of seizure types (Stephen et al, 1998) have been suggested to have additive or even synergistic effects.

A related question is at what dose a drug should be deemed non-efficacious and alternative treatment, whether substitution or combination therapy, be considered. The usual recommendation of escalating the dose to near-toxic levels in patients with persistent seizures (Perucca, 1996) assumes a positive dose-response relationship. Direct evidence supporting this approach is lacking. Such controversies concerning management approach arise largely because the "natural history" of newly diagnosed epilepsy in response to treatment is not well understood. This problem will be further discussed in Part I; Section 4.

The clinical properties of the AEDs relevant to the studies described in this thesis will be outlined below.

3.3.4 Established antiepileptic drugs

3.3.4.1 Phenobarbital

PB is the oldest among the currently licensed AEDs (see Part I; Section 3.1) and is indicated for the treatment of both partial and GTCS (Brodie and Dichter, 1996). Despite

being similarly efficacious, PB is less well tolerated than CBZ and PHT, mainly due to its sedative, cognitive and behavioural side effects (Mattson et al, 1985). PB is the archetypal enzyme inducer and so can accelerate the metabolism of a range of lipid soluble drugs (Patsalos and Duncan, 1993). For these reasons, it is rarely prescribed as a first-line AED for adults with newly diagnosed epilepsy in the industrialised world, although its low cost is relevant to its widespread use in developing countries.

3.3.4.2 Phenytoin

PHT is also effective against partial and GTCS. It is one of the few drugs in clinical use whose pharmacokinetics change from first to zero order at therapeutic dosage (Thomson and Brodie, 1992). To avoid toxicity, frequent monitoring of the plasma level is required. PHT is an enzyme inducer and a target for enzyme inhibitors (Brodie and Dichter, 1996). It can produce a range of neurotoxic symptoms and long-term cosmetic changes (gum hypertrophy, acne, hirsutism, facial coarsening). PHT is also teratogenic.

3.3.4.3 Carbamazepine

CBZ is effective against partial and GTCS, but can exacerbate myoclonic and absence seizures (Brodie and Dichter, 1996). Superior efficacy of CBZ (Mattson et al, 1992) as monotherapy for partial seizures over PHT and VPA has not be consistently demonstrated (Callaghan et al, 1985; Richens et al, 1994; Heller et al, 1995; De Silva et al, 1996). Its neurotoxic profile has been highlighted in comparative monotherapy studies (Brodie et al, 1995; Chadwick et al, 1998; Brodie et al, 1999a; Chadwick, 1999). Rash is common (10%) and may necessitate withdrawal of treatment. Other potentially serious idiosyncratic reactions include hepatotoxicity and blood dyscrasia. CBZ induces its own metabolism as well as that of other lipid-soluble drugs and is a target for inhibitors (Brodie, 1992). It is also teratogenic.

3.3.4.4 Sodium valproate

VPA is effective in patients with all types of seizures, with particular value in the IGEs (Brodie and Dichter, 1996). There is accumulating evidence to support a synergistic effect with LTG across a range of seizure types (Panayiotopoulos et al, 1993; Ferrie and Panayiotopoulos, 1994; Veggiotti et al, 1994; Brodie et al, 1997b; Pisani et al, 1999). VPA is easy to use and does not require routine monitoring of plasma concentration. It is generally well tolerated. Weight gain may be troublesome in some patients. An association with polycystic ovarian syndrome and hyperinsulinaemia in young women has been suggested recently (Isojärvi et al, 1993), although the overall impact is likely to be small and other predisposing factors may play a role (Stephen et al, 2000). Hepatotoxicity is rare (Dreifuss et al, 1987), but dose-related tremor and thrombocytopaenia are more common. *In utero* exposure to VPA increases the risk of neural tube defects to 1-2% (Crawford et al, 1999). VPA inhibits the metabolism of other AEDs, particularly PHT, PB, CBZ and its epoxide, and LTG (McKee and Brodie, 1994).

3.3.5 New antiepileptic drugs

The majority of the newer AEDs have been licensed only as adjunctive therapy, with the exception of LTG and OXC which are also available as monotherapy in the UK. GBP is approved as monotherapy in other parts of the world, while VGB can be used as monotherapy for infantile spasm. Many possess a diverse mechanistic profile (Table 4). Compared with their older counterparts, the newer AEDs tend not to affect hepatic metabolising enzymes, and are thus less prone to pharmacokinetic interactions (Brodie, 1992). There is also a growing impression that some of the more modern drugs, e.g. LTG and GBP, are more tolerable in their neurotoxicity profile (Kalviainen et al, 1996; Brodie,

1999). There are still insufficient monotherapy data in humans to confirm lack of teratogenicity with the newer AEDs (Morrell, 1996).

3.3.5.1 Lamotrigine

LTG has a wide spectrum of activity (Brodie, 1996). It is not sedative and generally well tolerated. It has demonstrated similar efficacy but better tolerability than PHT (Steiner et al, 1994) and CBZ (Brodie et al, 1995) in double-blind monotherapy trials in patients with partial and GTCS. There is evidence supporting an additive or synergistic effect when LTG is combined with VPA or TPM (Part I; Section 3.3.3). Rash occurs particularly in those receiving VPA, but the risk can be reduced by low dosage introduction and slow titration (Guberman et al, 1998). Animal and human data so far suggest low teratogenic potential (Crawford et al, 1999), although longer observation is required for confirmation. Drug interactions occur only with other AEDs.

3.3.5.2 Gabapentin

GBP is effective against partial-onset seizures and has few side effects (Dichter and Brodie, 1996). It was particularly well tolerated in add-on trials (Marson et al, 1997) and in monotherapy comparisons with CBZ (Chadwick et al, 1998) and LTG (Brodie et al, in press). No pharmacokinetic interactions with other drugs have been reported with GBP. Weight gain has been reported at high dosage (DeToledo et al, 1997).

3.3.5.3 Oxcarbazepine

OXC has a similar extent and range of efficacy to CBZ as monotherapy (Dam et al, 1989), but causes fewer idiosyncratic reactions and neurotoxic side effects (Shorvon, 2000). It is as effective for partial seizures with or without generalisation as PHT (Bill et al, 1997; Guerreiro et al, 1997) and VPA (Christie et al, 1997), although it was clearly better

tolerated than the former. OXC selectively induces the metabolism of female sex hormones, necessitating high oral contraceptive dosage (Fattore et al, 1999).

3.3.5.4 Topiramate

Although not always tolerated, TPM is highly efficacious for patients with refractory epilepsy (Marson et al, 1997; Stephen et al, 2000). It has a broad spectrum of action, being effective against partial-onset seizures as well as primary GTCS (Wilson and Brodie, 1996). Side effects include fatigue, paraesthesia and weight loss, less commonly word-finding difficulties, and rarely renal calculi. Its metabolism is induced by PHT and CBZ (Dichter and Brodie, 1996), and it selectively induces the metabolism of the hormonal components of the oral contraceptive pill (Rosenfeld et al, 1997).

3.3.5.5 Tiagabine

TGB is effective for treatment of partial seizures with or without secondary generalisation (Leach and Brodie, 1998). Its major practical drawback in clinical use is a short elimination half-life of 6-8 hours, which is further reduced by enzyme-inducing AEDs, such as PHT and CBZ, necessitating thrice-daily dosing in many patients (Wilson and Brodie, 1996).

3.3.5.6 Vigabatrin

VGB is approved as adjunctive treatment for partial seizures with or without secondary generalisation (Brodie and Dichter, 1996), but was not as efficacious as CBZ as monotherapy for partial seizures in adults in a large randomised study (Chadwick, 1999). Psychiatric complications including depression and psychosis have long been recognised (Sander et al, 1991). However, it is the recent concern over the high incidence of visual field defects associated with long-term VGB treatment (Miller et al, 1999) that has

relegated it to a drug of last choice, although it is still regarded to have particular value against infantile spasms (Appleton et al, 1999).

3.4 Surgical treatment of epilepsy

Modern epilepsy surgery dates back over 100 years to the publication of Sir Victor Horsley's classic paper (Horsley, 1886). In the ensuing years it has been met with a fluctuation in enthusiasm. The current wave of interest in epilepsy surgery began in the late 1980s and can be attributed largely to technical advances in neuroimaging and video EEG monitoring, improvements in surgical technique, and a better understanding of the anatomical and pathophysiological bases of epilepsy (Engel, 1996b).

The majority of operations for epilepsy are intended to be "curative", although less commonly "palliative" procedures are also carried out (Wieser, 1998). The goal of the former is seizure-freedom through complete resection of the seizure-generating focus. This type of surgery can be offered to patients with surgically remediable syndromes, the prototype of which is MTLE (see Part I; Section 1.4.2; Engel, 1996b). With modern techniques, 70-90% of patients with MTLE become seizure-free after anteromesial temporal lobectomy (Engel, 1999), with surgical mortality rate close to 0, and significant morbidity (e.g. hemiparesis, hemianopia) less than 5% (Wieser, 1998). Patients with partial seizures due to discrete structural lesions, such as glial tumours, may also be regarded as having surgically remediable syndromes. In a "palliative" procedure (e.g. corpus callosotomy, multiple subpial transection), the seizure focus is not resected, but rather the pathways important for the spread of epileptiform discharges are disrupted in order to ameliorate the seizure tendency (Wieser, 1998).

Presurgical evaluation aims to delineate the epileptogenic zone to be resected and to demonstrate that its removal will not cause additional unacceptable neurological or cognitive deficits. The extent of investigations depends on the specific procedure to be employed. In most cases, they include interictal and long-term video EEG monitoring to localise epileptic excitability, high quality MRI to identify structural abnormalities, functional imaging with PET or SPECT, and neuropsychological testing, including intracarotid injection of amobarbital to identify the laterality of language and memory function tests. In some cases, invasive intracranial EEG recording is needed (Engel, 1996b).

Despite its efficacy and safety for appropriately selected patients, surgical treatment for epilepsy is heavily underutilised (Engel, 1999). It is estimated that half of the patients who are refractory to AED treatment may benefit from epilepsy surgery (Engel and Shewmon, 1993). In the USA, this translates into 100 000 to 200 000 patients, and an extra 5000 to 10 000 are being added per year. However, in 1990 only 1500 surgical procedures were performed (Engel, 1999). Such underutilisation may in part be due to the perceived invasiveness and cost of presurgical evaluation. In addition, epilepsy surgery is still largely considered by physicians to be a "last resort" for medically refractory epilepsy, which is itself poorly understood and ill defined. With the arrival of so many new AEDs, no patient will be able to try every agent (as monotherapy and in combination) in a lifetime. At what stage of the disease process does epilepsy become "refractory", and are there clinical features that can help to identify these patients early so that surgery can be considered without relentless futile trials of AEDs? These questions can only be answered through understanding the natural history of treated epilepsy, which has not been well documented.

3.5 Other non-pharmacological treatment of epilepsy

Vagus nerve stimulation (VNS) is a new, non-pharmacological approach to epilepsy treatment. It was approved in 1997 in the USA and subsequently in Canada and the European Union countries as adjunctive treatment for refractory partial-onset seizures in adults and adolescents. In this system, the left vagus nerve is stimulated via a bipolar lead connected to a programmable signal generator implanted subcutaneously in the patient's left upper chest (Schachter and Saper, 1998). The mechanism of action of VNS is unknown, and how it complements AED therapy is still unclear. Other forms of "stimulation therapy" for epilepsy under development include stimulation of the centromedian thalamic nucleus (Velasco et al, 1995) and transcranial magnetic stimulation (Ziemann et al, 1998).

4 Natural history of epilepsy

4.1 Untreated epilepsy

Until very recently, the prognosis of epilepsy had traditionally been viewed with pessimism. Hippocrates believed that seizures beginning in adulthood lasted until death (Tempkin, 1971). Gowers conceded that "the spontaneous cessation of seizures is an event too rare to be anticipated in any given case" (1881). However, recent cross-sectional population-based studies of untreated patients in rural areas of developing countries suggest spontaneous remission may occur in a significant number of patients. One such study in Ecuador found 46% of the untreated cases were in long-term remission (Placentia et al, 1992). Data from other developing countries such as Nigeria, Ethiopia, China and India, where AED therapy is usually not available, reported similar prevalence rates to the developed world where most patients are treated, again suggesting that the disease may remit spontaneously (Sander, 1993). However, such evidence is uncontrolled and only circumstantial. The true spontaneous remission rate can only be ascertained accurately by

randomly assigning newly diagnosed patients to treatment or no treatment, which is difficult to justify ethically given that effective therapy has been available since 1857 and AED therapy is usually commenced as soon as epilepsy is diagnosed in developed countries.

4.2 Treated epilepsy

Most of the data concerning the natural history of epilepsy, therefore, relate to its response to treatment. Until the late 1960s, Gowers' gloomy opinion was echoed by many subsequent studies, which reported remission rate with treatment between 10 and 30% (Cockerell, 1996). These studies, however, were small, hospital-based and retrospective. Most modern large-scale prospective studies, including only newly diagnosed patients followed up for long periods, tended to suggest a remission rate of 60-80% (Table 7).

4.2.1 Population-based studies

The two most influential community-based studies were performed in Rochester, Minnesota, USA, and the UK, respectively. In the Rochester Epidemiology Project, patients are identified from a medical records-linkage system which includes all medical contacts of residents of Olmsted County, Minnesota, since 1935 (Annergers et al, 1996). Results primarily concerning the incidence, prevalence and causes of seizures and epilepsy have been published in a series of reports, covering the periods from 1935 to 1967 (Hauser and Kurland, 1975), 1940-1980 (Hauser et al, 1991), 1935 to 1984 (Hauser et al, 1993), 1980 to 1984 (Zarrelli et al, 1999), and as summary articles for the 50-year period (Hauser et al, 1996; Annegers et al, 1996). The issues of remission and early prognostic factors were explored in two reports (Annegers et al, 1979; Shafer et al, 1988). In the initial analysis, the probability of being in remission for 5 years at 20 years after diagnosis (i.e. terminal remission) was 70% (Annegers et al, 1979). This was revised to 75% in the more

Table 7. Terminal remission data from selected studies.

Study	No. of patients	Population of study	Median follow-up years	Years in remission	% in remission*
Elwes et al, 1984	106	Hospital	5.5	2	79
Shafer et al, 1988	432	Community	17	5	66
Collaborative, 1992	280	Hospital	4	1	70
Cockerell et al, 1995b	564†	Community	7	5	68
Sillanpaa et al, 1998‡	176	Hospital	28	1	80

* Percentage in remission at median follow-up.

† Definite epilepsy.

‡ Children only.

recent report (Shafer et al, 1988). Predictors of a better outcome were absence of early-life brain damage, absence of generalised epileptiform activity on EEG, and no history of GTCS. The predictive value of these factors, however, was weak (Shafer et al, 1988).

In the National General Practice Study of Epilepsy, patients with newly diagnosed or suspected epilepsy were identified from 275 general practices throughout the UK between 1984 and 1987 and followed up for a median of 7.1 years (Cockerell et al, 1995). Cumulative remission rates (i.e. acturial estimates of percentage seizure-free at any time during follow-up) and terminal remission rates for various durations at various time intervals were calculated. Among the 564 patients with "definite" epilepsy, the chance of achieving 5-year remission after 9 years from diagnosis was 68%. Analysis of prognostic factors had not been published at the time of writing of this thesis.

4.2.2 Relationship between outcome and AED treatment

Although these two projects have provided valuable estimation of the probability of remission in relation to time from diagnosis, analysis of prognostic factors was often performed irrespective of treatment status. No study has specifically addressed the relationship between outcome and the course of AED treatment, i.e. to examine outcome with "drug treatment" as the variable instead of "time". Undoubtedly, it is important to the patient to know his/her likelihood of seizure-freedom at at various time points after the onset of seizures or diagnosis. However, the great majority of patients, at least in the developed countries, would be started on AED treatment when a diagnosis of epilepsy is made. From a more practical point of view, it would also be useful to gauge the chance of successful treatment with the first AED chosen. Perhaps more importantly, when the first drug fails to control the epilepsy, as is often the case, little is known about the proportion

of patients responding to subsequent monotherapy, combination therapy, and whether there is any difference in efficacy between these two treatment strategies.

Such poor documentation of progress in response to treatment has resulted in confusion and lack of consensus over the definition of "refractory" epilepsy (Perucca, 1998; Regesta and Tanganelli, 1999) and has hindered the development of a strategic approach to management. The usual "routine" management consists of monotherapy at diagnosis, followed by combinations of AEDs if it fails. When epilepsy remains uncontrolled, it is labelled "pharmacoresistant", "intractable" or "refractory". Some of these patients will be enrolled in experimental drug studies, some offered epilepsy surgery, or more recently, a vagus nerve stimulator. But with at least 15 major AEDs available, the lifetime of a patient will not be sufficient to allow a trial of all options. How many trials of single AEDs should be employed before the patient is treated with duotherapy (Schmidt and Gram, 1995)? At a practical level, how many trials of drugs, either singly or in combinations, have to fail before the seizure disorder can be regarded as "refractory" and potentially "curative" surgery considered? At what stage during the course of the disease does epilepsy become "pharmacoresistant"? Currently, the diagnosis of "refractory" epilepsy is *a posteriori*, i.e. after it has failed to respond to "appropriate" AED treatment. But are some epilepsies refractory *de novo* (Shorvon, 1990), and can they be identified early by their clinical features so these patients may be targeted for epilepsy surgery or effective combination drug treatment without unnecessary delay?

A better understanding of how epilepsy responds to AED treatment is needed to allow a more accurate assessment of the factors influencing prognosis and help the formulation of a more strategic approach to management. Such knowledge cannot be obtained by retrospective or case-control studies which are prone to inclusion bias, but only by

studying newly diagnosed patients prospectively. A series of clinical studies carried out for this purpose are described in Part II.

5 Biological basis of refractory epilepsy

Various factors may contribute to pharmacoresistance, some of which may be unrelated to the biology of the disease ("pseudoresistance"), such as poor compliance of medication, inappropriate lifestyle (e.g. excessive alcohol intake), inappropriate choice of AED or drug regimen, and inappropriate assessment of response (Perucca, 1998). These possibilities should be considered and rectified before a patient's epilepsy is labelled "refractory".

5.1 *De novo* or an evolving process?

Whether refractory epilepsy evolves over time or is present at the outset remains hotly debated. Proponents of the former point to the unfavourable clinical prognostic factors of epilepsy, such as a large number of pre-treatment seizures, a long interval between the first seizure and initiation of treatment, early onset of seizures, history of complex febrile seizures, and a declining interval between seizures in untreated patients (Table 8; Reynolds, 1995; Regesta and Tanganelli, 1999). At the other end of the clinical spectrum, where seizure-free patients withdraw from AED therapy, the shorter the remission duration, the more likely is the patient to relapse (Medical Research Council, 1991). These observations may either be due to a progression of the causative disorder or to secondary epileptogenesis, i.e. a deterioration of the seizure disorder caused by the epileptic activity itself (Morrell, 1985; 1991). Recent long-term outcome studies of patients undergoing temporal lobectomy for refractory epilepsy support the suggestion that secondary epileptogenesis at sites distant to the lesion may develop with uncontrolled seizures (Eliashiv et al, 1997). The contention that "seizures beget seizures" (Gowers, 1881) can be substantiated by the experimental model of kindling, whereby electrical stimulation at

Table 8. Poor clinical prognostic factors demonstrated in some studies (Shorvon, 1996; Perucca, 1998; Pegesta and Tanganelli, 1999).

Characteristics of seizures	Symptomatic epilepsies
Early onset of seizures	Complex febrile seizures
Large number of pretreatment seizures	Learning disabilities
Long interval between first seizure and treatment	Progressive neurological disorders
High seizure frequency	Family history of epilepsy
Partial or mixed seizure types	Multifocal EEG paroxysms
Persistence of seizures	
History of status epilepticus	

what is initially a subconvulsive level in an animal, subsequently becomes sufficient to induce seizures after repeated application (Löscher and Schmidt, 1988). Based on similar lines of reasoning, it is argued that potentially "curative" epilepsy surgery should be performed early to avoid the seizures becoming more severe with time with resultant irreversible cognitive decline and psychosocial disability (Engel, 1998b; 1999).

On the other hand, there is no strong clinical data to suggest that kindling is relevant to human epilepsy (Shinnar and Berg, 1996). High pretreatment seizure number may simply be a marker for rather than the cause of poor prognosis. In a recent Italian multicentre study, treatment of the first seizure, although reducing the risk of a second seizure, did not improve the long-term probability of seizure-freedom (Musicco et al, 1997). In a study of children with epilepsy, initiation of treatment after 10 or fewer seizures did not influence the remission rate (Camfield et al, 1996). In rural Africa where patients may have been untreated for many years with numerous seizures, response to AED treatment was similar to patients who are treated early in developed countries (Feski et al, 1991; Watts, 1992). Randomised trials have shown that treatment with AEDs to prevent the occurrence of acute symptomatic seizures, such as febrile convulsions (Berg and Shinnar, 1997), post-traumatic seizures (Temkin et al, 1990) and after craniotomy (Foy et al, 1992), does not reduce the risk of subsequent epilepsy. Many of the poor prognostic factors, such as brain damage, EEG abnormalities, family history of epilepsy and specific syndromes, are present at the onset of the disease.

Lastly, it should be noted that much of the discussion on the role of repeated seizures in the generation of chronic epilepsy concerns patients at the onset of their disease and does not address the possibility of a progressive process in those who continue to have seizures despite AED treatment.

5.2 Mechanism of pharmacoresistance

5.2.1 The "usual suspects"

Given that regardless of the exact definition, up to one third of patients have drug-resistant epilepsy, it is surprising that little research has been carried out to address its biological mechanism. It is likely to be multifactorial and variable. Among the idiopathic epilepsies, prognosis is often determined by the underlying syndrome (Berg and Shinnar, 1997). There are, for instance, a number of devastating encephalopathic disorders in infancy for which no consistently effective treatment is available (Pellock, 1999). Certain structural abnormalities (e.g. MTS, CD) appear to be particularly pharmacoresistant (Semah et al, 1998; Regesta and Tanganelli, 1999), suggesting response to AED treatment may be influenced by the causative neuropathology.

Even for MTS which is the most extensively studied epilepsy-related pathology, the molecular basis of its pharmacoresistance is poorly understood. The "usual suspects" are abnormalities in neuronal network, neurotransmitter receptors and ion channels (Table 9). In addition to selective neuronal loss and gliosis in the CA1 and CA3 regions, the "sprouting" of mossy fibres which may be hyperexcitable is well recognised in both human HS and experimental models (Pringle et al, 1993). The "dormant basket cell" theory, which hypothesises that inhibitory neurons survive but are rendered hypofunctional by a loss of excitatory systems that normally evoke stimulation (Sloviter, 1991), is another attempt to explain the epileptogenicity of HS by plastic changes in neuronal circuitry (Lothman, 1994). Pharmacoresistance may be a result of alteration in neurotransmitter receptors. Changes in composition of inhibitory $GABA_A$ receptors (Brooks-Kayal et al, 1998) and increased expression of subclasses of excitatory glutamate receptors have been reported in animal models of MTLE and in man (Blümcke et al, 1999). With the

Table 9. Putative factors contributing to the biological basis of refractory epilepsy.

Syndromic classification e.g. childhood epileptic encephalopathies
Causative neuropathology e.g. mesial temporal sclerosis, cortical dysplasia
Hyperexcitable and disinhibited neuronal network reorganisation e.g. mossy fibre sprouting
Altered neurotransmitter receptors e.g. composition/functioning of GABA/glutamate receptors
Ion channelopathies e.g. sodium, calcium, potassium channels
Reactive autoimmunity e.g. autoantibodies against glutamic acid decarboxylase
Impaired antiepileptic drug penetration e.g. P-glycoprotein expression at blood-brain barrier
? Pharmacogenetic variations

GABA = γ-aminobutyric acid

increasing number of ion channelopathies being identified in human genetic epilepsies (Wallace et al, 1998; Steinlein, 1999) and animal epilepsy models (Cox et al, 1997; Bentar, 1999), it is possible they may also play a role in the mechanism of drug resistance. Epilepsy-related changes in the dynamics of calcium entry through voltage- and transmitter-gated channels and in intraneuronal calcium buffering have long been recognised (Blümcke et al, 1999).

5.2.2 Other potential factors

Although these studies may have pinpointed factors that contribute to the epileptogenicity or seizure susceptibility of a given syndrome, they do not address the mechanism of drug resistance. Why do some patients with, say, MTS remain refractory despite treatment with 4 AEDs while others with a seemingly identical lesion are seizure-free on a modest dose of a single drug? The potential role of immunological factors, drug penetration and genetics in the genesis of pharmacoresistance has not been fully explored.

Antibodies to the glutamate receptor subtype GluR3 are thought to cause Rasmussen's encephalitis, a rare form of epilepsy characterised by seizures, progressive neurological dysfunction and inflammatory histopathology (Andrews and McNamara, 1996). Antibodies directed against GM1 ganglioside, which are highly epileptogenic in a rat model, have been implicated in human epilepsy (Bartolomei et al, 1996). There have been recent case reports of elevated glutamic acid decarboxylase (GAD) autoantibodies in patients with refractory partial seizures (Martinelli et al, 1978; Solimena et al, 1988; Saiz et al, 1996; Nemni et al, 1994; Giometto et al, 1998). Autoantibodies to GAD, which catalyses the conversion of L-glutamic acid to GABA, may, theoretically, impair its activity and the subsequent production of GABA resulting in refractory epilepsy. Whether

elevated GAD autoantibodies contribute to drug resistance in epilepsy is examined by a case-control study in Section 5 of Part II.

One of the puzzling features of refractory epilepsy is its resistance to multiple AEDs, either singly or in combination, with varied modes of action. This characteristic suggests the operation of a universal mechanism affecting AEDs in general. One such possible candidate involves alterations at the blood-brain barrier (BBB). AEDs, by default, have to cross the BBB to exert their antiepileptic effect. The potential role of changes in the BBB at the epileptogenic zone in refractory epilepsy has not been investigated. Changes in the BBB, especially a non-specific "opening", during acute seizures have been described (Cornford and Oldendorf, 1986; Duncan and Todd, 1991; Cornford, 1999). However, little is known about changes in chronic epilepsy (Cornford, 1999). If the function of the BBB was enhanced around the epileptogenic focus by some mechanisms, AEDs might be prevented from entering their site of action, but could still penetrate the rest of the brain to cause neurotoxicity. The epilepsy might, therefore, display resistance to multiple AEDs with different mechanisms of action. This might also explain why symptomatic epilepsies (with structural lesions) are more drug-resistant than idiopathic epilepsies (usually genetic with presumably more diffuse changes).

Changes in mechanisms governing the access of AEDs to the seizure focus may potentially provide a unifying biological basis for pharmacoresistant, or "multidrug resistant", epilepsy. One such candidate mechanism is a family of drug transporters (Twentyman, 1997), the prototype of which is P-glycoprotein (P-gp) which is found in cerebral capillary endothelium and functions as a drug efflux pump, contributing to the integrity of the BBB (Van Asperen et al, 1997). The importance of the BBB and the potential role of P-gp in

the pathogenesis of pharmacoresistance in epilepsy will be further discussed and explored by a series of animal and human studies in Part III.

Diversity in an individual's response to drugs is well recognised not only in epileptology, but also across all medical disciplines. Much of this variation is now thought to be due to genetic differences (polymorphisms) which are studied under the heading of "pharmacogenetics" (Nebert, 1999). Traditionally, pharmacogenetics has focused on drug-metabolising genes as a determinant of individual susceptibility to drug toxicity (Ball and Borman, 1998; Evans and Relling, 1999). However, genetic polymorphisms may also influence a drug's efficacy in a given patient, a phenomenon now recognised in many fields of medicine, including asthma, Alzheimer's disease and coronary heart disease (Evans and Relling, 1999; Roses, 2000a). Pharmacogenetics has gained ever greater momentum with the completion of a "working draft" of the human genome (Macilwain, 2000). Pharmacogenetic profiling by DNA chips to personalise treatment so that the "right drug is given to the right patient" may soon be realised (Marshall, 1998; Wolf et al, 2000; Roses, 2000b). The potential impact of pharmacogenetics on epileptology is already being felt by the demonstration of greater sensitivity of mutated nicotinic acetylcholine receptors (which underlie autosomal dominant nocturnal frontal lobe epilepsy) to CBZ compared to wild type *in vitro* (Picard et al, 1999). Polymorphisms of the multidrug resistance (*MDR*) gene, which codes for P-gp, with functional changes affecting drug accumulation and metabolism *in vivo* have recently been reported (Hoffmeyer et al, 2000). It is possible that such genetic variation may explain in part the heterogeneous response to AED therapy, further underlining the importance to study whether P-gp plays a role in epilepsy.

6 Overall aims

The aims of the current project were:

(1) To ascertain the prognosis of epilepsy in newly diagnosed patients.

(2) To identify clinical features that allow early prediction of refractory epilepsy.

(3) To document progress in the response to AED therapy among patients with newly diagnosed epilepsy.

(4) To determine whether underlying pathology influences outcome.

(5) To determine whether GAD autoantibodies are associated with AED-resistance.

(6) To determine whether commonly used AEDs are substrates of P-gp.

(7) To determine what role, if any, P-gp plays in the genesis of AED-resistance.

Clinical studies carried out to examine (1) to (5) are described in Part II, while laboratory-based experiments exploring (6) and (7) are reported in Part III.

PART II

CLINICAL STUDIES

1 Natural history of treated epilepsy -- Early identification of refractory epilepsy

1.1 Introduction and aim

As discussed in Part I, Section 4, the major dilemma inherent to the sequential management approach of epilepsy lies in the imprecise understanding and definition of pharmacoresistance (Perucca, 1998). Although the recent improvement of surgical outcome and the dramatic expansion of AED therapy have substantially increased the chance of better seizure control, how to target the new treatment modalities is unclear. The introduction of 9 new AEDs since 1989 with more to come means that drug choice has been dramatically widened and the number of possible combinations is now almost limitless. How many trials of single AEDs should be employed before the patient is treated with duotherapy? How many AEDs, either singly or in combination (and in how many combinations), have to fail before the seizure disorder can be recognised as "refractory" and surgery considered? At what stage during the course of the disease does epilepsy become "pharmacoresistant" to AED treatment? Perhaps more importantly, are there clinical features that allow prediction of subsequent "refractoriness"?

To address these questions, a longitudinal outcome study was conducted to delineate the "natural history" of treated epilepsy. In particular, this study aimed to identify clinical features associated with poor response to therapy. Early identification of subsequent refractoriness may enable better targeting of combination AED therapy or surgery to "high risk" patients.

1.2 Methods

1.2.1 Patients

The study included consecutive unselected patients in whom epilepsy was diagnosed and AED therapy begun at the Epilepsy Unit, Western Infirmary, Glasgow, Scotland between 1

January 1984 and 31 December 1997. Patients were enrolled into the study when they fulfilled the inclusion criteria and followed up prospectively at the Epilepsy Unit. The Epilepsy Unit is one of the major referral centres for the management of patients with epilepsy in Glasgow. Most of the patients were referred to the Epilepsy Unit by general practitioners with a minority (8%) from the hospital's accident and emergency department (McKee et al, 1990). The majority of patients reside in Glasgow, although some patients travel to the clinic from other parts of the west of Scotland.

A case file was created for each patient and stored on-site at the Epilepsy Unit, allowing instant access when required. At the first visit, demographic and clinical information was collected from the patient, and any witness, using a structured questionnaire. A general physical and neurological examination was performed. Additional investigations were carried out as clinically indicated. Surface EEG, either routine or sleep-deprived, was performed and reported by neurophysiologists and neuroimaging, particularly CT or MRI, was carried out by radiologists with interest in neuroradiology. Information obtained from the history, examination and investigations was used to classify the patient's seizure and epilepsy type according to the guidelines of the ILAE (Part I; Sections 1.1 and 1.2). During subsequent visits, the degree of seizure control, any side effects of medication, serum drug levels, and other relevant information were recorded in an ongoing database for subsequent analysis.

1.2.2 Approach to treatment

For each patient diagnosed to have epilepsy, the appropriate AED was chosen taking into account such factors as the types of seizure and epilepsy, patient characteristics, side effects, and interaction profiles of the available drugs (Part I; Section 3.3). Some patients volunteered to participate in randomised AED trials, in which case the drug remained

unknown to both the clinician and the patient during the study period. Protocols for all drug trials were approved by the Ethics Committee of the Western Infirmary, and all patients or their parents or legal guardians provided written informed consent.

Patients were subsequently evaluated at the clinic every 4 to 6 weeks for the first 6 months and at least every 4 months thereafter. If medical attention was necessary between the scheduled appointments, the patients or their general practitioners could call the Epilepsy Unit by a dedicated telephone line. At each follow up visit, clinical information and the response to AED therapy were recorded in an ongoing prospective database (Tobias et al, 1994). Compliance was monitored at the clinic with the help of on-site measurement of serum drug levels (McKee et al, 1993).

Drug dosage was titrated according to recommended schedules (Brodie and Dichter, 1996; Dichter and Brodie, 1996) and adjusted as clinical circumstances dictated, with particular attention paid to efficacy and tolerability. Monotherapy was used where possible, as is recommended practice (Part I; Section 3.3.2). Treatment was changed to another drug if seizures remained uncontrolled or if the patient developed an idiosyncratic reaction or intolerable side effects. Patients whose epilepsy was the result of a possibly removable structural abnormality, such as MTS, tumour, or AVM, were referred for surgical evaluation (Part I; Section 3.4) at another centre.

1.2.3 Definitions and statistical analysis

For the purpose of assessing any potential correlation between various clinical factors and outcome, patients were divided into two groups according to whether or not they were seizure-free during follow-up. The extent of seizure control was assessed at the time of the patient's last clinic visit. Patients were considered seizure-free if they had not had seizures

of any type for at least one year (i.e. terminal remission). Response to treatment was classified as seizure-free, failure due to inadequate seizure control (lack of efficacy) or adverse events (idiocyncratic reactions such as rash and hepatotoxicity, or intolerable side-effects), or withdrawal of treatment due to reasons unrelated to efficacy or tolerability such as concern about potential adverse effects, planning a pregnancy, and a change of mind. A change of treatment regimen was defined as substitution of an existing drug or addition of a new drug.

The Chi-square test was used for comparisons of categorical data and the Mann-Whitney test for non-parametric continuous data. Non-parametric analyses were performed because the variables (e.g. age) were not normally distributed in the two groups and not equal in variance. Relative risks and their 95% confidence intervals for both significant and non-significant differences were calculated (Altman, 1991). The Chi-square test for trend was used to assess the effect of pre-treatment seizure number on outcome. Potential interaction between factors was examined by logistic regression analysis. All statistics tests were two-tailed. Statistical calculations were performed with use of Minitab for Windows (Release 11.21) software.

1.3 Results

1.3.1 Patient demographics

Overall, 629 of the 3,209 patients who were referred to the clinic between 1st January 1984 and 31st December 1997 were not being treated at the time of referral. They included patients who had not previously been diagnosed to have epilepsy and those in whom AED treatment had been withdrawn. Eight patients died from a variety of causes during treatment, 74 did not return for follow-up after the first AED was started, and 22 were excluded because of uncertainty about the diagnosis or persistent non-compliance with

treatment. The remaining 525 patients (52% male) constituted the study group. Among them, 470 patients had never received AED therapy. The median duration of follow-up was 5 years (range, 2 to 15 years), and 90% of the patients attended the clinic for at least 3 years. The median age at referral was 29 years (range, 9 to 93 years), and the median age at onset of epilepsy was 26 years (range, <1 to 92 years). There was no significant difference in sex (relative risk [RR] 0.92, 95% confidence interval [CI] 0.80 to 1.04), age at referral (p=0.63) or the onset of seizures (p=0.56), prevalence of a family history of epilepsy (RR 0.98, 95% CI 0.84 to 1.15), or history of febrile convulsions (RR 0.97, 95% CI 0.72 to 1.35) between the group that became seizure-free and the group with uncontrolled epilepsy (Table 10).

1.3.2 Effects of previous treatment

At the last clinic visit, 333 (63%) patients were seizure-free (Figure 4). There was no significant difference (RR 0.87, 95% CI 0.69 to 1.11) in eventual seizure-free rate between the 55 patients who had previously received one or more AEDs (56%) and those not previously treated (64%). The 55 patients who had previously received AED treatment were included in the analysis for other factors associated with refractory epilepsy with the exception of the analysis exploring response to first AED.

1.3.3 Epilepsy classification

One hundred and forty patients (27%) were classified as having idiopathic epilepsy, 150 (28%) as symptomatic epilepsy and 235 (45%) as cryptogenic epilepsy. The proportion of patients with symptomatic or cryptogenic epilepsy who continued to have seizures during treatment was higher than in the patients with idiopathic epilepsy (40% vs. 26%, p=0.004; relative risk 1.5; 95% CI 1.1 to 2.1). There was no difference in the proportions of

Table 10. Clinical characteristics of 525 patients with epilepsy who became seizure-free or had persistent seizures while receiving antiepileptic drug therapy.

Characteristics		Seizure-free n (%)	Uncontrolled n (%)
Total number	All patients	333 (63)	192 (37)
Sex	Male	157 (47)	102 (53)
	Female	176 (53)	90 (47)
Age at onset (years)	Median	25	26
	Range	<1 – 92	1 – 75
Age at referral (years)	Median	27	31
	Range	9 - 93	13 – 76
Family history	Positive	74 (22)	44 (23)
	Negative	259 (78)	148 (77)
Febrile convulsion	Positive	16 (5)	10 (5)
	Negative	317 (95)	182 (95)
Type of epilepsy	Idiopathic	103 (31)	37 (19)
	Symptomatic	86 (26)	64 (33)
	Cryptogenic	145 (43)	90 (47)
Number of seizures at baseline	20 or fewer	242 (73)	98 (51)
	Over 20	91 (27)	94 (49)

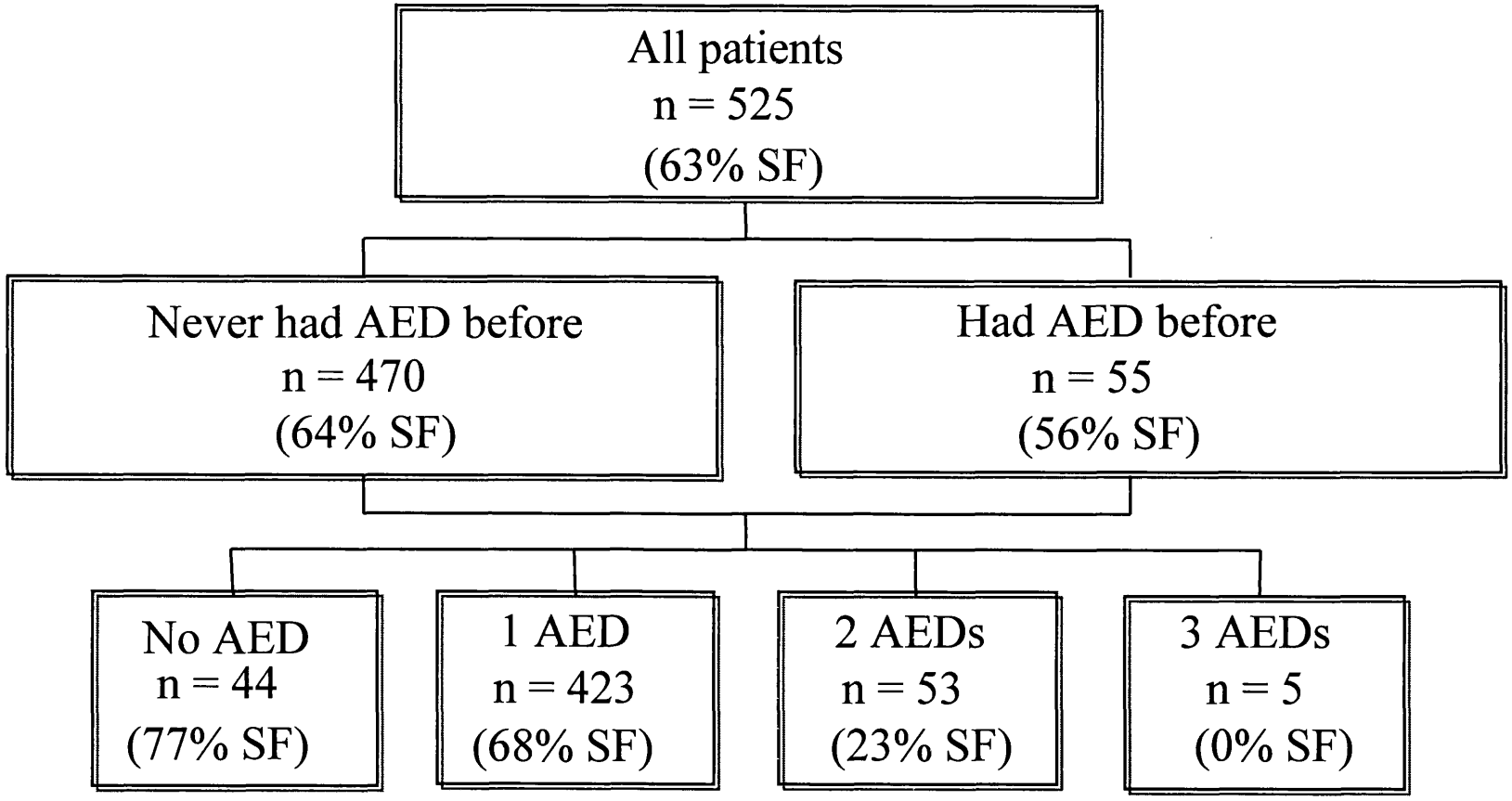


Figure 4. Outcome in 525 patients after commencement on antiepileptic drug (AED) therapy receiving various number of drugs. The status of the patient at the time of the last clinic visit is given in parentheses. SF = seizure-free.

patients with symptomatic and cryptogenic epilepsy who continued to have seizures (43% vs. 38%; RR 1.11, 95% CI 0.27 to 1.42, respectively).

1.3.4 Pre-treatment seizure number

There was a significant linear trend in the proportion of patients with uncontrolled epilepsy in relation to the number of pre-treatment seizures (χ^2=32.7, p<0.001; Figure 5), even after excluding patients with only one seizure (χ^2=35.2, p<0.001). Epilepsy was uncontrolled in 94 of the 185 patients (51%) who reported more than 20 seizures before initiation of therapy (p<0.001; relative risk 1.8, 95% CI 1.4 to 2.2) as compared with 98 of the 340 patients (29%) who had 20 seizures or less. Logistic regression analysis revealed no significant interaction between type of epilepsy and number of seizures before treatment.

1.3.5 Antiepileptic drug therapy

Four hundred and twenty-three patients (81%) were being treated with a single AED at the last clinic visit, with 289 receiving an established drug (CBZ 155, VPA 125, PHT 8, ESM 1) and 134 taking one of the newer AEDs (LTG 99, GBP 15, OXC 7, TGB 9, TPM 3, VGB 1). There was no significant difference in seizure-free rates between the groups (67% vs. 69%; RR 0.98, 95% CI 0.85 to 1.12). Fifty-three patients were being treated with 2 AEDs, of whom only 12 (23%) were seizure-free (Figure 4). None of the 5 patients receiving 3 AEDs were seizure-free. Forty-four patients had chosen not to continue AED therapy, some after a period of remission (39%), some due to side-effects (48%) and the remainder for personal reasons (13%). Thirty-four (77%) of these patients had been seizure-free for more than a year.

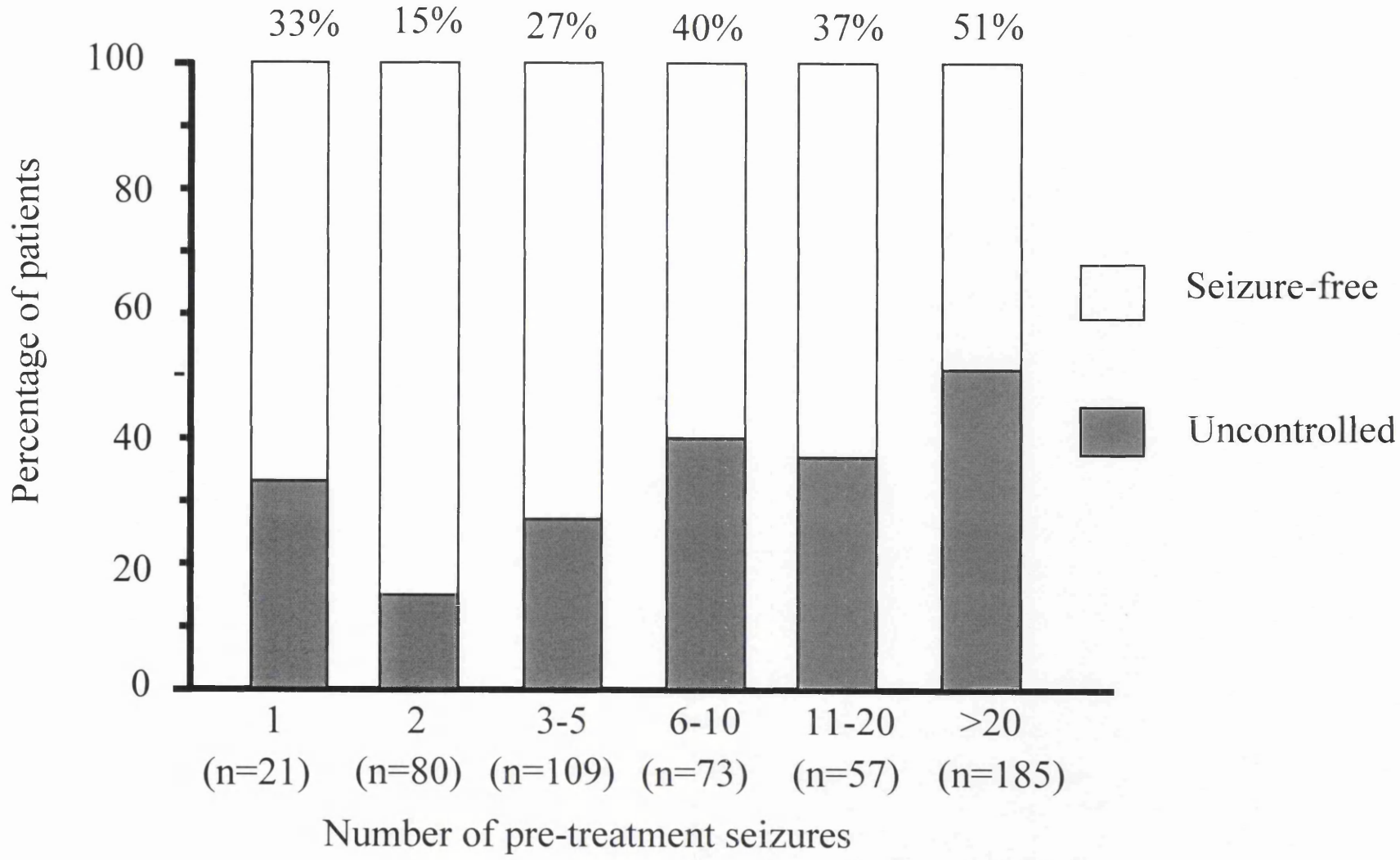


Figure 5. Outcome in patients according to the number of seizures before treatment. The percentages of patients with uncontrolled epilepsy are shown on top of the bars ($p<0.001$ for the comparison with patients who were seizure-free).

One hundred and ninety-five patients were enrolled in double-blind trials comparing an established AED with a new drug. In the 104 patients (53%) who completed such a study (established drugs: CBZ 38, VPA 13; new drugs: LTG 30, GBP 5, OXC 5, TGB 12, FBM 1), there was no significant difference in seizure-free rate between the patients receiving the established and new drugs (established drug 71%, new drug 66%; RR 1.07, 95% CI 0.8 to 1.4). All but four of the patients who received a new drug continued to be seizure-free while receiving the same drug after the study ended. Ninety-one patients (47%) did not complete the study, the majority (59%) because of side-effects. One hundred and twenty (62%) of the 195 patients who participated in single-drug trials became seizure-free, a figure which was similar to the rest of the cohort, suggesting no bias in the patient selection for these studies.

1.3.6 Response to the first drug and subsequent outcome

Among the 470 patients who had never received an AED, 301 (64%) became seizure-free. Two hundred and twenty-two (47%) were controlled with their first AED which was an established drug in 151 patients and a new drug in 71 patients (Figure 6), of whom 15 remained seizure-free after discontinuation of the drug. The seizure-free rates were the same in both drug groups (RR 1.01, 95% CI 0.82 to 1.23). Sixty-seven patients (14%) became seizure-free during treatment with a second or third drug. Only 12 patients (3% of total) were controlled with two drugs (Table 11). The probability of attaining seizure-freedom decreased progressively with successive AED regimens, and rapidly so after the first two regimens (Figure 7).

Treatment with the first AED was unsuccessful in 113 patients due to poor seizure control, 69 because of intolerable side-effects, 29 because of idiosyncratic reactions, and 37 due to other reasons. Only 79 of these 248 patients (32%) in whom the first drug was ineffective

Table 11. Success of antiepileptic drug regimens in 470 patients with previously untreated epilepsy.

Variable	No.	(%)
Seizure-free on the first drug	222	(47)
Seizure-free during continued therapy with first drug	207	(44)
Remained seizure-free after discontinuation of first drug	15	(3)
Seizure-free on the second drug	61	(13)
Seizure-free during monotherapy with second drug	41	(9)
Remained seizure-free after discontinuation of second drug	20	(4)
Seizure-free on the third drug	6	(1)
Seizure-free during therapy with two drugs	12	(3)
Total	301	(64)

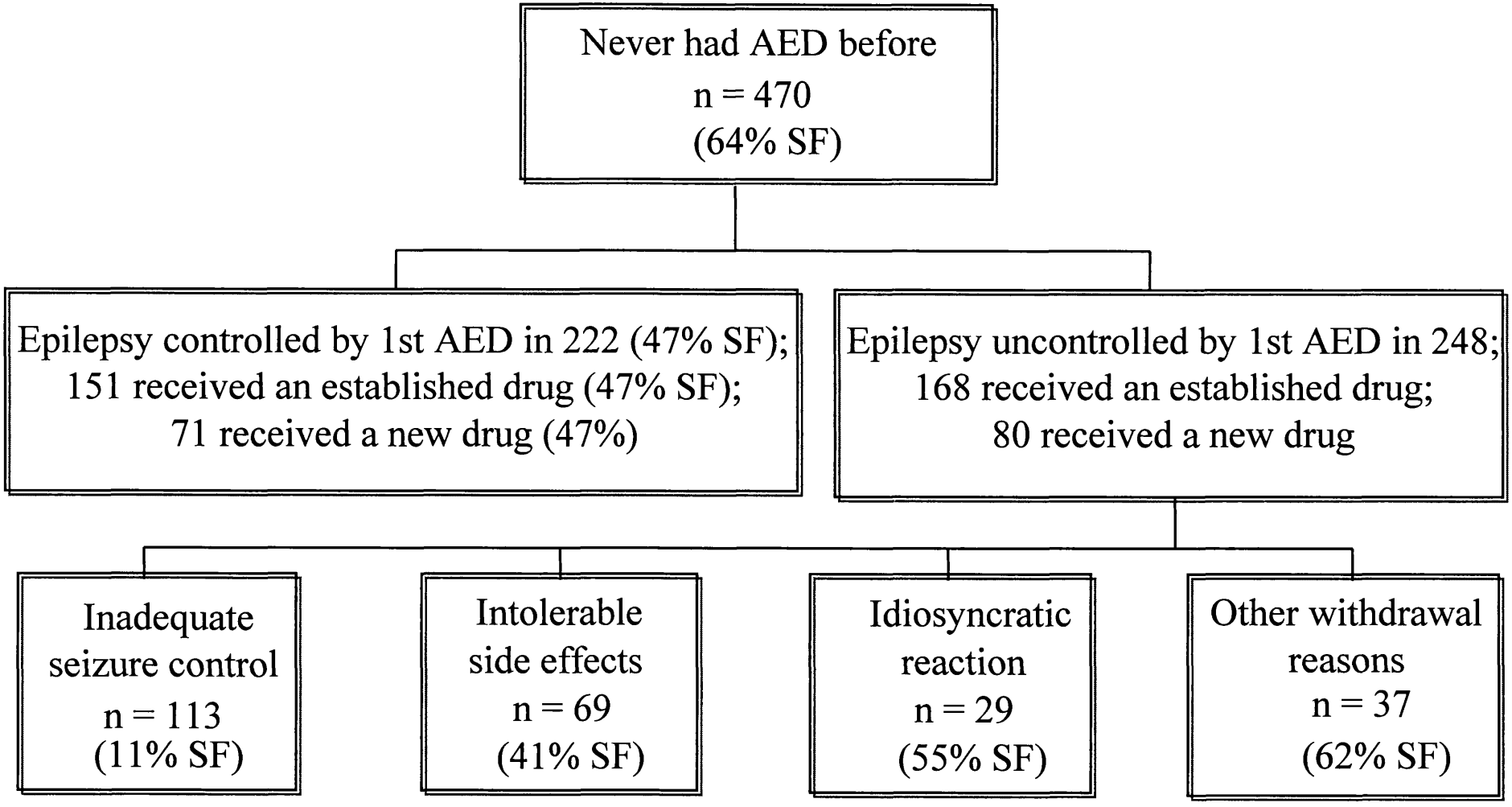


Figure 6. Response to the first antiepileptic drug (AED) and final outcome in 470 previously untreated patients. The status of patients at the time of the last clinic visit is given in parentheses. SF = seizure-free

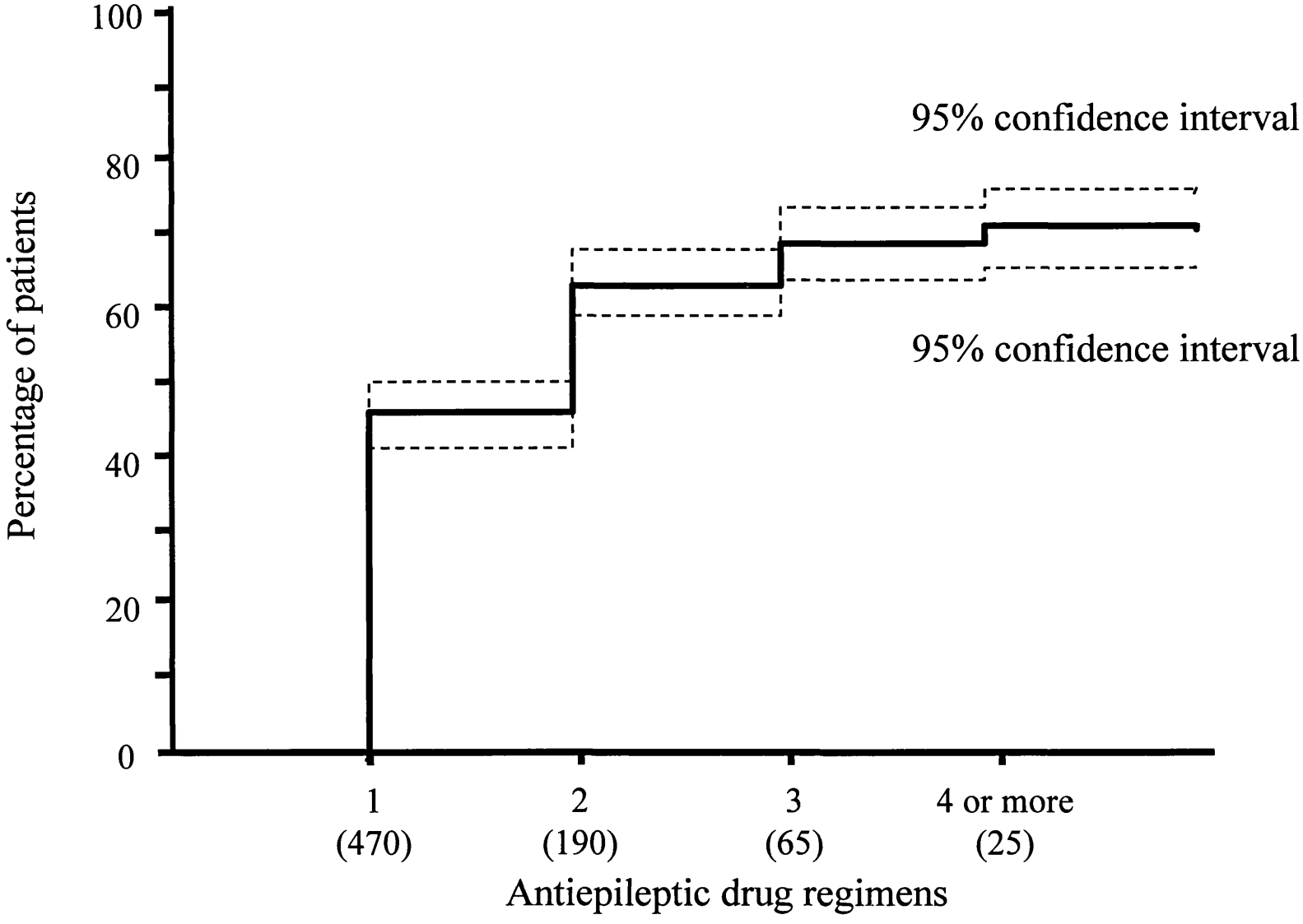


Figure 7. Kaplan-Meier survival plot of probability of attaining seizure-freedom with successive epileptic drug regimens in previously untreated patients. Number of patients at risk given in parentheses.

subsequently became seizure-free. The outcome among these patients was strongly associated with the reason for initial failure (χ^2=57.0, p<0.001; Figure 6). Fifty-five percent of the patients with an idiosyncratic reaction became seizure-free, as did 41% of the patients with intolerable side effects, but only 11% of those with inadequate seizure control subsequently became seizure-free.

1.4 Discussion

The overall remission rate of epilepsy in this study of 63% was comparable to that in several recent hospital-based studies (Table 7). Consistent with previous reports (Annegers et al, 1979; Shafer et al, 1988; Sillanpaa, 1993; Berg et al, 1996; Sillanpaa et al, 1998), patients with a known or probable structural cerebral abnormality were 1.5 times more likely to have uncontrolled epilepsy. The reason for this is unclear. Potential differences in response pattern to AED treatment between patients with idiopathic and symptomatic/cryptogenic epilepsy will be further examined in Part II; Section 2. Whether limitation of AED access to the epileptogenic lesion contributes to the genesis of pharmacoresistance in symptomatic epilepsy will be explored in Part III.

A high number of seizures before treatment was a poor prognostic indicator, an observation that has also been made previously (Collaborative Group, 1992; Sillanpaa, 1993). Whether high pretreatment seizure number is the cause of or merely a marker for severe seizure disorders is hotly debated (Part I; Section 5.1). Although a causal relationship between a high number of pre-treatment seizures and later intractibility may be supported by the experimental phenomenon of kindling, whereby an animal subsequently develops seizures after initially subconvulsive electrical stimulation (Reynolds, 1995), clinical studies have failed to suggest significant relevance of kindling in human epilepsy (Shinnar and Berg, 1996). Studies comparing outcome between early and delayed

initiation of AED treatment (Camfield et al, 1996; Musicco et al, 1997), and reports from developing countries on long-term prognosis of patients newly commenced on therapy after a long history of untreated seizures (Feski et al, 1991; Watts, 1992), do not support the notion of "seizures beget seizures" (Gowers, 1881). Many authors, therefore, believe that a high number of seizures before treatment is the result, rather than the cause, of the pathophysiological changes that later manifest as refractory epilepsy (Berg and Shinnar, 1997). However, it remains to be tested whether kindling occurs in specific epilepsy syndromes, for example MTLE on which the experimental model is based, and which is suggested by a growing body of evidence to be progressive in nature (Engel, 1999).

From a more practical point of view, the finding that many patients were seizure-free while taking a single AED is in agreement with the consensus that monotherapy is a realistic goal for most patients (Brodie and Dichter, 1996; Brodie and French, 2000) and, indeed, that the overall prognosis of epilepsy is good (Anonymous, 1997). This is reinforced by the observations that 47% of patients became seizure-free during treatment with their first drug and that 77% of those who stopped treatment remained seizure-free. Epilepsy may run a benign course and remit spontaneously in some patients (Keranen and Riekkinen, 1993).

The rates of remission were similar in patients treated with an established AED and those treated with a new drug. In randomised, double-blind trials comparing CBZ with LTG, there was no difference in efficacy between the two drugs, although fewer side effects and lower drop-out rates were reported among patients treated with LTG (Brodie et al, 1995; Brodie et al, 1999a).

An early response to drug therapy confers a favorable prognosis (Annegers et al, 1979; Elwes et al, 1984; Collaborative Group, 1992; Sillanpaa et al, 1998). Results from this

study suggest that the response to the first AED is also a powerful prognostic factor. This factor was particularly useful among patients in whom treatment failure was due to inadequate efficacy: only 11% of such patients later became seizure-free, as compared with 41% of the patients who had intolerable side-effects and 55% of those with an idiosyncratic reaction. The probability of attaining seizure-freedom declined progressively with successive AED regimens, and few became seizure-free after failure of two regimens. For all patients in whom treatment with the first AED was unsuccessful, 14% became seizure-free when changed to another drug but only 3% became seizure-free while taking a combination of two drugs.

These observations support the hypothesis that difficult-to-control epilepsy may be present *de novo* rather than evolve over time, since the clinical characteristics are apparent early in the course of disease. These patients are more likely to have underlying structural cerebral abnormalities, to present with more than 20 seizures, and to not respond to their first AED. Such theoretical considerations have practical implications for devising a more strategic approach to the management of epilepsy. From a pragmatic point of view, these data suggest that patients may be considered to have difficult-to-control epilepsy after failure of two first-line AEDs. It may be argued that this may be the case even as soon as the first AED fails due to inadequate efficacy (as opposed to intolerable side effects) since only 1 in 9 of such patients became seizure-free in the long-term.

This consideration reinforces the assertion that for patients with correctable structural abnormalities, epilepsy surgery should be considered as soon as two first-line drugs fail (Engel, 1996; 1999). In selected groups of patients, this approach can render up to 90% of patients seizure-free (Part I; Section 3.4). For the majority of patients not suitable for "curative" resective surgery, AEDs remain the mainstay of treatment. The finding that

only 3% of patients became seizure-free on more than one drug highlights the need to combine drugs more effectively. There is accumulating evidence to suggest that certain combinations, such as VPA and LTG (Brodie et al, 1997b), have additive or even synergistic effects, raising the possibility of a mechanistic approach to "rational polytherapy" (Part I; Section 3.3.3).

1.5 Conclusion

In conclusion, subsequent "refractoriness" may be predicted from clinical characteristics early in the course of the disease, allowing strategic targeting of surgery or effective polytherapy. Whether early performance of surgery or institution of "rational" polytherapy prevents the development of refractory epilepsy remains to be tested. A multicentre randomised trial to compare early and late surgery for patients with MTLE is soon to start (Engel, 1999), and a study comparing substitution and combination therapy after failure of the first AED is already underway in Glasgow.

From a management standpoint, many crucial questions remain. For instance, when should treatment with the first AED be deemed to have failed and alternative therapy, either substitution or polytherapy, be considered? Secondly, in patients without correctable structural abnormality, when and how should combination of AEDs be used? These issues are addressed in Part II; Sections 2 and 3 respectively.

2 Natural history of treated epilepsy -- Effectiveness of first ever antiepileptic drug

2.1 Introduction and aim

The natural history of newly diagnosed epilepsy in response to treatment has not been well documented (Part I; Section 4.2). Long-term outcome studies (Part II; Section 1) and randomised comparative trials (Mattson et al, 1992; Richens et al, 1994; Brodie et al, 1995; Beydoun, 1997) suggest that fewer than 50% of patients become seizure-free on the first AED. As shown in Part II; Section 1, failure on the first AED due to lack of efficacy is associated with poor subsequent outcome. Effectiveness encompasses both efficacy and tolerability (ILAE, 1998), but the importance of the latter is often inadequately assessed in randomised, particularly regulatory, studies which are not designed to address the everyday practical use of the drug (Chadwick, 1997). In patients with persistent seizures, it is unclear at what dosage the drug should be deemed non-efficacious and when alternative treatment, such as a second drug or a combination of 2 drugs, should be considered (Part I; Section 3.3.3). The usual recommendation of escalating the dose to near-toxic levels in patients with persistent seizures (Perucca, 1996) assumes a positive dose-response relationship. Direct evidence supporting this is lacking.

Although randomised controlled trials are essential to establish the efficacy of a new AED for regulatory purposes, whether their results can be extrapolated to clinical practice has been questioned (Walker and Sander, 1997). Observational studies may assist the translation of trial data into everyday usage (Mant, 1999; Pocock and Elbourne, 2000; Stephen et al, 2000). This study aimed to explore the interaction among efficacy, tolerability and overall effectiveness of the first ever AED in patients with newly diagnosed epilepsy. These data facilitated observations on dose-response relationships with the commonly used first choice AEDs in this setting.

2.2 Methods

2.2.1 Patients

The study included patients in whom epilepsy was diagnosed and treatment initiated at the Epilepsy Unit in the Western Infirmary in Glasgow, Scotland between 1st January 1984 and 31st December 1997. Only those who had never received AED therapy were included in the analysis. During the first visit, a structured questionnaire was used to collect clinical information from the patients and any witnesses to the seizure. Investigations, in particular surface EEG and neuroimaging (Part I; Section 2.2), were carried out as clinically indicated. Epileptic seizures and syndromes were classified using information obtained from the history, physical examination and investigations according to the ILAE guidelines (Part I; Sections 1.2 and 1.3). The classification for each patient represented the final status at the time of analysis.

Treatment principles and follow-up arrangements were as described in Part II; Section 1.2.2.

2.2.2 Statistical analysis

Patients were divided into 2 groups for purposes of analysis according to whether or not they became and remained seizure-free on their first ever AED. They were considered to be seizure-free if they had no auras or seizures of any type for at least one year. Response to medication was classified as: seizure-free; able to tolerate the medication but with persistent seizures (inadequate seizure control); change of treatment due to adverse events (including side-effects and idiosyncratic reactions); or withdrawal of treatment for reasons unrelated to efficacy or tolerability such as concern about potential adverse events, planning a pregnancy, and a change of mind.

The Chi-square test and Fisher's exact test were used for comparisons of categorical data, and the Mann-Whitney test for non-parametric continuous data. Multiple nominal logistic regression was employed to examine the potential interaction between factors such as the type of epilepsy and the antiepileptic drug prescribed, and to assess the difference between individual drugs in terms of treatment response. All statistical tests were two-tailed. Calculations were made using Minitab for Windows (Release 11.21) software.

2.3 Results

2.3.1 Patient demographics

Four hundred and seventy (51% male) previously untreated patients with newly diagnosed epilepsy entered the study. The median follow-up period was 5 years (range, 2 to 15 years), with 91% of patients attending the clinic for at least three years. The median age at referral was 29 years (range, 9 to 93 years), and the median age at onset of epilepsy was 26 years (range, 1 to 92 years). One hundred and nineteen patients (25.3%) were classified as having idiopathic epilepsy, 138 (29.4%) as having symptomatic epilepsy, and 213 (45.3%) as having cryptogenic epilepsy (Table 12).

2.3.2 Epilepsy type

Overall, 47% patients became seizure-free on the first ever AED. A higher proportion of patients with localisation-related epilepsy (symptomatic or cryptogenic) came off their first drug due to intolerable side-effects (17% compared to 8.5% in patients with idiopathic epilepsy; p=0.025; relative risk 2.00; 95% confidence interval (CI) 1.06 to 3.78) and a lower proportion became seizure-free (43.5% compared to 58% in patients with idiopathic epilepsy; p=0.007; relative risk 0.75; 95% CI 0.62 to 0.91). There was no significant difference in other outcome parameters between the two patient groups (Figure 8). There

Table 12. Classification and aetiologies of epilepsy in newly diagnosed patients according to whether their epilepsy was controlled or uncontrolled by the first ever antiepileptic drug.

Aetiology/syndrome	Controlled	Uncontrolled	Total	(%)
Idiopathic	69	50	119	(25.3)
Tonic-clonic seizures	48	37	85	(18)
Juvenile myoclonic epilepsy	18	4	22	(5)
Juvenile absence epilepsy	1	2	3	(<1)
Childhood absence epilepsy	0	1	1	(<1)
Unclassified	2	6	8	(2)
Symptomatic	60	78	138	(29.4)
Post-stroke	17	22	39	(8)
Post-traumatic	18	17	35	(7)
Cerebral atrophy	8	14	22	(5)
Tumour	4	6	10	(5)
Perinatal insult	3	4	7	(1)
Mesial temporal sclerosis	2	2	4	(1)
Cortical dysplasia	1	2	3	(1)
CNS infection	2	0	2	(<1)
Arteriovenous malformation	0	1	1	(<1)
Congenital malformation	0	1	1	(<1)
Demyelination	0	1	1	(<1)
Multiple causes	5	8	13	(3)
Cryptogenic	93	120	213	(45.3)
Total	222	248	470	(100)

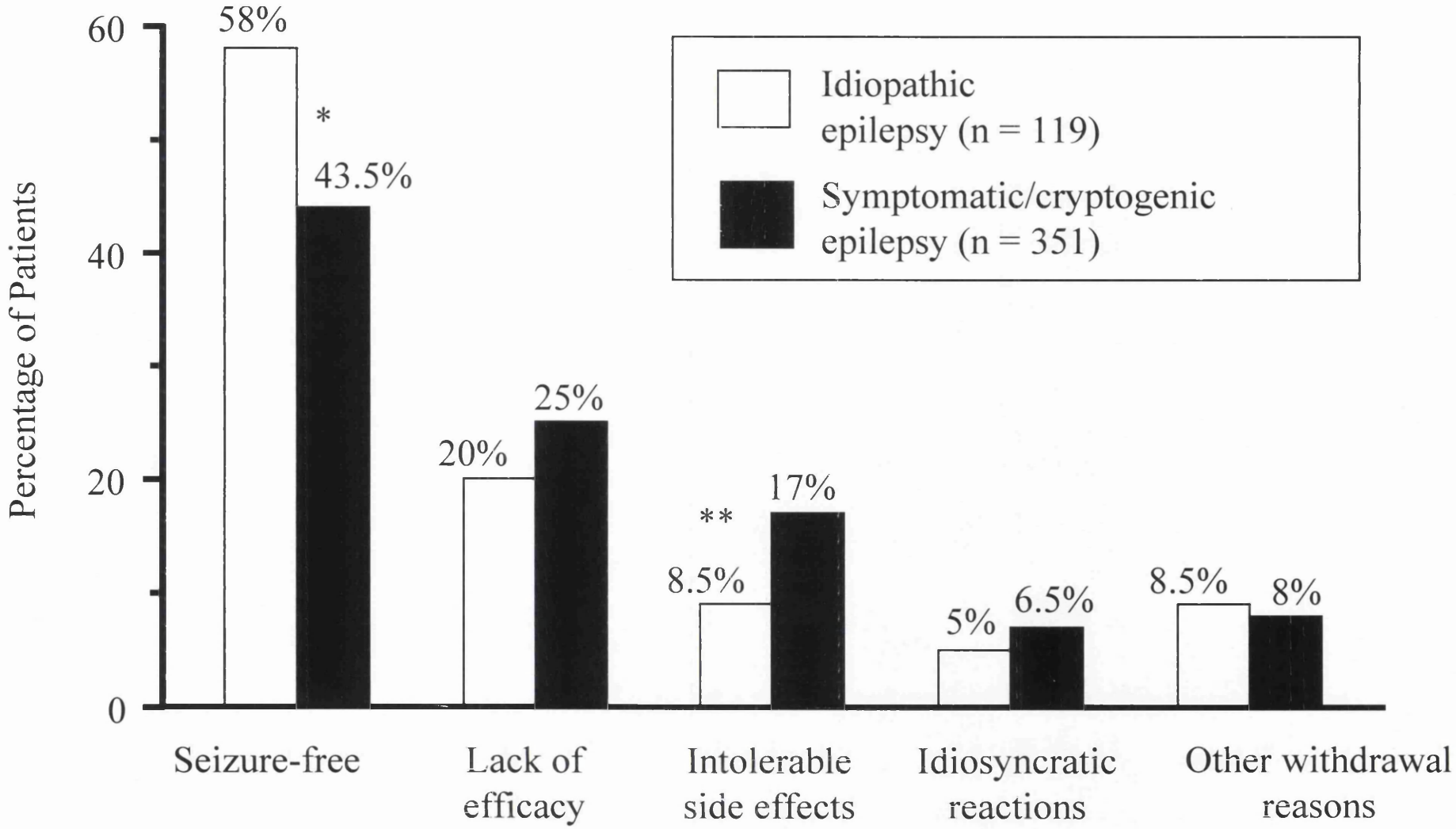


Figure 8. Response of previously untreated patients with newly diagnosed epilepsy to the first antiepileptic drug. *p<0.01, **p<0.05.

was no difference in response to the first AED between the symptomatic and cryptogenic patient groups, and among the major specific aetiological categories (Table 12).

2.3.3 Dose-response relationship

The most frequently prescribed first AEDs were CBZ (n=212), VPA (n=101) and LTG (n=78), which accounted for treatment in 83% of the patient population. Twenty-seven patients received TGB, 19 GBP, 17 OXC, and 10 FBM, all within randomised double-blind comparative trials. The remaining 6 patients were treated with PHT. Among patients treated with CBZ, VPA or LTG, those who became seizure-free (CBZ, $p<0.0001$; VPA, $p<0.01$; LTG, $p<0.001$), and those who had to change treatment due to intolerable side-effects (CBZ, $p<0.0001$; VPA, $p<0.01$) took lower doses than those with inadequate seizure control (Table 13). For all 3 drugs (Figure 9), over 90% of seizure-free patients required only a moderate daily dose (800mg CBZ or less, 1500mg VPA or less, 300mg LTG or less). Little additional seizure control was achieved as the dose was further increased. The majority of withdrawals due to adverse events also occurred at or below these doses (Figure 10; CBZ: 98%, VPA: 100%, LTG: 75%).

Patients with idiopathic and symptomatic/cryptogenic epilepsy took similar mean/median daily doses of VPA (n=56, 1040mg/1000mg and n=45, 1202mg/1000mg, respectively). There was also no significant difference in LTG doses between the 2 groups (idiopathic: n=20, 207.5mg/200mg; symptomatic/cryptogenic: n=58, 234.9mg/200mg), but the former group was receiving lower doses of CBZ (n=29, 469mg/400mg) compared to the latter group (n=183, 568mg/600mg; $p=0.05$). However, for all 3 drugs, there was no significant difference in drug dosages between the 2 patient groups within each response category.

Table 13. Median (interquartile range) final daily doses (mg/day) of first antiepileptic drug in different response groups.

Response	Carbamazepine	Sodium valproate	Lamotrigine
Seizure-free	600 (400-600)*	1000 (800-1000)†	187.5 (150-200)¶
Inadequate seizure control	800 (600-800)	1500 (1000-2000)	300 (200-400)
Intolerable side-effects	400 (275-600)*	800 (500-1200)†	300(150-375)
Idiosyncratic reaction	200 (200)	--	100 (50-150)
Withdrew for other reasons	600 (400-800)	1250 (1000-1875)	175 (150-200)
All patients	600 (400-600)	1000 (825-1500)	200 (150-300)

*$p<0.0001$ compared to patients on carbamazepine with inadequate seizure control.

†$p<0.01$ compared to patients on sodium valproate with inadequate seizure control.

¶ $p<0.001$ compared to patients on lamotrigine with inadequate seizure control.

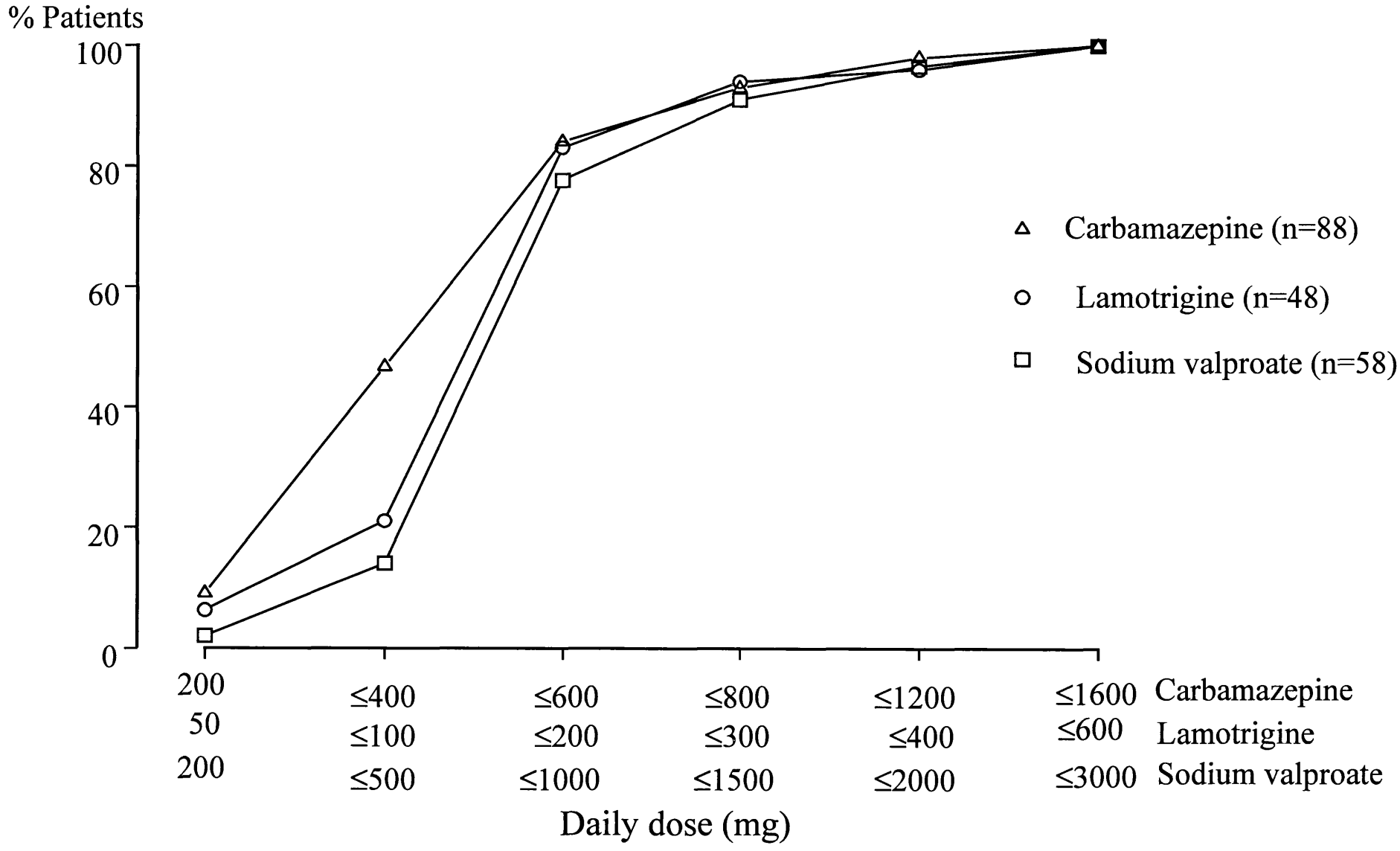


Figure 9. Daily doses of first antiepileptic drugs in seizure-free patients.

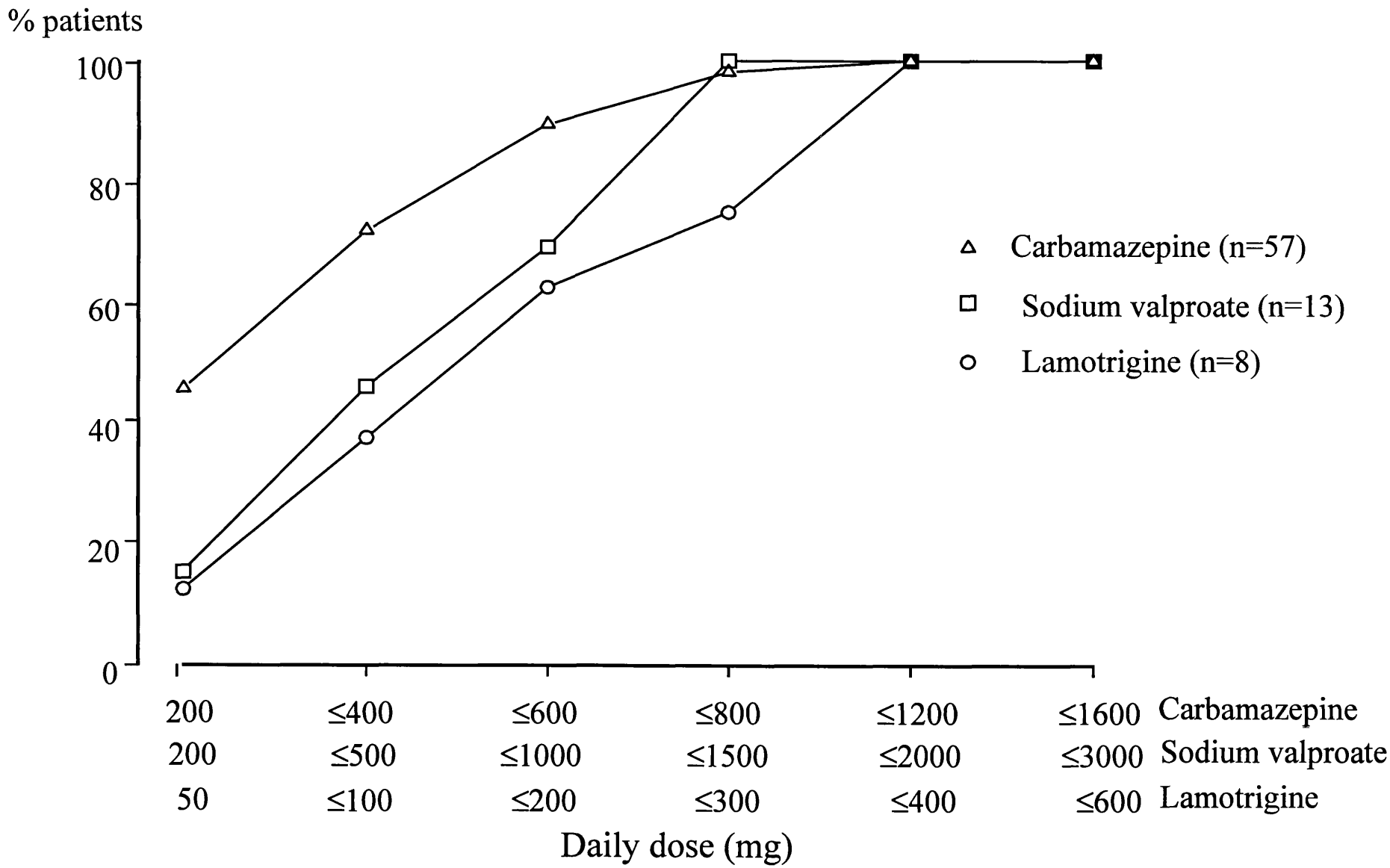


Figure 10. Daily doses of first antiepileptic drugs associated with adverse events necessitating drug withdrawal.

2.3.4 Individual drugs

There was no significant difference in the proportion of patients able to tolerate each of the 3 major AEDs but who had inadequate seizure control, suggesting similar efficacy (Table 14). However, more patients treated with CBZ changed medication due to adverse events compared to those given VPA or LTG and, consequently, fewer became seizure-free (CBZ vs. VPA: p=0.02, odds ratio 0.43, 95% CI 0.20 to 0.89; CBZ vs. LTG: p=0.002, odds ratio 0.27, 95% CI 0.12 to 0.62). There was no difference in outcome between patients treated with VPA and LTG.

Although the proportions of patients receiving the drugs were different among the idiopathic and localisation-related groups, nominal logistic regression analysis revealed no significant interaction in terms of treatment outcome between epilepsy type and the AED prescribed, suggesting that they acted as independent factors. Nevertheless, separate sub-analyses were undertaken for the two patient groups. A lower proportion of patients with idiopathic epilepsy (all with GTCS as the only seizure type) given CBZ became seizure-free than those treated with VPA or LTG (CBZ vs. VPA: 41% vs. 64%, p=0.04; CBZ vs. LTG: 41% vs. 80%, p=0.007), again largely because they had a higher withdrawal rate due to adverse events (CBZ vs. VPA: 28% vs. 11%, p=0.05; CBZ vs. LTG: 28% vs. 0%, p=0.01). Among patients with localisation-related epilepsy, a higher proportion of patients treated with CBZ changed treatment due to adverse events than those prescribed LTG (CBZ vs. LTG: 27% vs. 14%, p=0.04), although the difference in seizure-free rates did not reach statistical significance (CBZ vs. LTG: 41.5% vs. 55%, p=0.07). Among patients with symptomatic/cryptogenic epilepsy, there was no significant difference in seizure-free rate between those treated with CBZ and VPA (41.5% vs 49%).

Table 14. Response to the first antiepileptic drug in newly diagnosed epilepsy.

	Carbamazepine		Sodium valproate		Lamotrigine	
	No.	(%)	No.	(%)	No.	(%)
Seizure-free*	88	(41.5)	58	(57)	48	(61.5)
Inadequate seizure control	53	(25)	26	(26)	20	(26)
Changed due to adverse events*	57	(27)	13	(13)	8	(10)
Withdrew due to other reasons	14	(6.5)	4	(4)	2	(2.5)
Total	212	(100)	101	(100)	78	(100)

* Carbamazepine vs. sodium valproate, p=0.02; carbamazepine vs. lamotrigine, p=0.002.

The most common adverse event necessitating drug withdrawal was rash (Table 15), which developed more often with CBZ than VPA (p=0.001). The differences in rash rate, however, between CBZ and LTG and between VPA and LTG were not statistically significant. Patients not tolerating CBZ often complained of more than one symptom. For VPA and LTG, the most frequent side effect leading to drug withdrawal was headache. Non-neurological side effects were uncommon with both these AEDs (Table 15).

2.4 Discussion

This study aimed to assess the response to the first AED. Newly diagnosed patients, mostly adults, were followed up prospectively as per standard clinical practice. Nearly 50% became seizure-free on the first ever AED, and over 90% did so at moderate doses. The marginal benefits in terms of additional seizure control by increasing the dose further were relatively small. The majority of withdrawals due to adverse events also occurred at low doses.

Examination of response to AEDs at different dose levels had not been conducted in randomised studies employing flexible dosing (Richens et al, 1994; Brodie et al, 1995; Heller et al, 1995; De Silva et al, 1996; Brodie et al, 1999a). A similar pattern emerged for CBZ, VPA and LTG. Although "standard" maintenance daily doses as monotherapy up to 2000mg for CBZ, 3000mg for VPA, and 500mg for LTG are recommended (Brodie et al, 1996; BNF, 2000), over 90% of seizure-free patients in this study required only a moderate amount of AED, beyond which few more became controlled. This lack of a positive dose-response relationship beyond the "placebo dose" has been noted in randomised monotherapy studies with other new AEDs employing 2 or 3 fixed doses (Beydoun, 1997; Chadwick et al, 1998).

Table 15. Adverse events among patients not tolerating the first antiepileptic drug.

Adverse events	Carbamazepine (n=212)		Sodium valproate (n=101)		Lamotrigine (n=78)	
	No.	(%)	No.	(%)	No.	(%)
Rash	22	(10)	0	(0)	3	(4)
Headache	12	(6)	4	(4)	2	(3)
Dizziness	10	(5)	1	(1)	0	(0)
Nausea/vomiting	9	(4)	2	(2)	0	(0)
Dysphoria	6	(3)	2	(2)	1	(1)
Somnolence	6	(3)	1	(1)	1	(1)
Fatigue	4	(2)	0	(0)	0	(0)
Ataxia	2	(1)	0	(0)	0	(0)
Cognitive disturbance	1	(<1)	1	(1)	0	(0)
Change in mood	1	(<1)	0	(0)	2	(3)
Tremor	0	(0)	2	(2)	0	(0)
Visual disturbance	1	(<1)	1	(1)	0	(0)
Insomnia	1	(<1)	0	(0)	0	(0)
Itch	1	(<1)	1	(1)	0	(0)
Impotence	1	(<1)	0	(0)	0	(0)
Hyponatraemia	1	(<1)	0	(0)	0	(0)
Total adverse events*	78		15		9	
Patients withdrawn+	57	(27)	13	(13)	8	(10)

* Some patients reported more than one adverse event.

+ Carbamazepine vs. sodium valproate, p=0.02; vs. lamotrigine, p=0.002.

Since AED doses are often progressively increased in patients with uncontrolled seizures, it is perhaps not surprising that these patients received a higher average dose compared to the seizure-free patients. Patients intolerant of AED treatment also took a lower average dose compared to the responding patients, and the majority of withdrawals due to adverse events occurred at low doses. The reasons for such extreme difference in response (seizure-freedom to intolerability at relatively low doses) is poorly understood, but may reflect underlying neuropathologies and pharmacogenetic factors (Part I; Section 5.2). Effectiveness (efficacy and tolerability) of an AED may, therefore, be determined in most patients when a moderate dose is reached. Escalating the dose to potentially toxic levels will improve seizure control in only a few more patients, while many more may have been delayed or even denied the opportunity of more effective treatment with alternative or combination therapy (Part I; Section 3.3.3) or epilepsy surgery (Part I; Section 3.4).

Patients with symptomatic/cryptogenic epilepsy were less able to tolerate AED treatment compared to those with idiopathic epilepsy. CBZ was less well tolerated than VPA or LTG. Although more patients with localisation-related epilepsy were given CBZ than the other 2 AEDs, as would be expected in routine practice, the differences in tolerability among the 3 drugs were more marked for patients with idiopathic epilepsy. This suggests that epilepsy type and choice of AEDs affected outcome independently, as was also supported by the multiple logistic regression analysis.

Tolerability of individual AEDs appeared to be an important factor in determining overall effectiveness. VPA and LTG were better tolerated than CBZ in this cohort of newly diagnosed patients. Similar findings have been reported in randomised trials comparing CBZ and VPA, and CBZ and LTG, involving previously untreated patients (Part I, Section 3.3.4.3; Richens et al, 1994; Brodie et al, 1995; Heller et al, 1995; De Silva et al, 1996;

Brodie et al, 1999a). The Veterans Affairs study (Mattson et al, 1992), which concluded superior control of complex partial seizures for CBZ compared to VPA, had many different patient and study characteristics from the present study population, including the enrolment of previously treated and mainly older males and the usage of high VPA doses (Reynolds et al, 1993).

Comparisons between the 3 drugs in this observational study inevitably suffer from potential confounding factors, including bias in selection of AEDs for individual patients. In addition, although CBZ is effective against primary generalised tonic-clonic seizures (Part I; Section 3.3.4), it is possible that it was prescribed to some patients with absence or myoclonic seizures which it may exacerbate (Brodie and Dichter, 1996) and which might not have been apparent at initial presentation. This may have contributed to its lower seizure-control rate compared to VPA and LTG which have a wider range of activity (Part I; Sections 3.3.4.4 and 3.3.5.1), and highlights the advantage of broad-spectrum AEDs for newly diagnosed patients (Brodie, 1999). A randomised trial in patients with well-defined seizure types is required to confirm the differences in seizure-free rate among the 3 drugs observed in this study.

The study described in Part II; Section 1 suggested that patients with a known or probable structural cerebral abnormality are more likely to have refractory epilepsy compared to those with idiopathic epilepsy which is presumed to have a genetic origin. This study suggests that a reduction in their ability to tolerate AED therapy may contribute to this difference in prognosis. Although these patients received a higher average dose of CBZ compared to those with idiopathic epilepsy, the majority of adverse events necessitating drug withdrawal occurred at low doses. Therefore, difference in doses, and the disproportionate distribution of the drugs across the patient groups, could not wholly

account for the difference in tolerability between them. Variation in tolerability of AEDs between patients with different seizure types has also been noted in randomised studies (Richens et al, 1994). Why this should be the case is unclear.

2.5 Conclusion

Approximately 50% of newly diagnosed patients will become seizure-free on the first AED. Over 90% will do so at modest doses. Effectiveness (efficacy and tolerability) of an AED may be determined in most patients when a moderate dose is reached. Alternative or combination therapy should be considered early. Tolerability is as important a factor as efficacy in determining drug choice (Mattson et al, 1985; Brodie et al, 1995; Beydoun, 1997; Chadwick, 1997; Brodie et al, 1999a; Brodie and Kwan, 2001).

3 Natural history of treated epilepsy -- Substitution or add-on after the first drug fails?

3.1 Introduction and aim

When and how combination of AEDs should be used in patients unresponsive to monotherapy is not known. Results from long-term outcome studies, including that carried out at the Epilepsy Unit described in Part II; Sections 2, as well as randomised trials (Mattson et al, 1992; Richens et al, 1994; Brodie et al, 1995; Beydoun, 1997) suggest that fewer than 50% of newly diagnosed patients will become seizure-free on the first AED. An alternative drug is unavoidable when the patient develops intolerable adverse events, but when seizures persist despite a sufficient dose being tolerated, it is unclear whether or not substitution should be tried before a combination of two drugs is used (Schmidt and Gram, 1995).

This controversy arises largely because the natural history of newly diagnosed epilepsy in response to treatment is not well understood (Part I; Section 4). Since the advantages of single- over multiple-drug treatment, particularly in terms of side effect profile, were demonstrated by a series of studies in the late 1970s and early 1980s (Part I; Section 3.3.2), monotherapy has rightly become the established principle for managing new onset epilepsy. However, the explosion of new AEDs in the past decade with their different mechanisms of action and generally better tolerability, has raised the possibility of effective and safe combinations for patients unresponsive to monotherapy (Part I; Section 3.3.3). The effectiveness of substitution and add-on therapy after treatment with the first AED has failed was examined in a cohort of newly diagnosed patients.

3.2 Methods

3.2.1 Patients

The study included unselected patients in whom epilepsy was diagnosed and treatment initiated at the Epilepsy Unit in the Western Infirmary in Glasgow, Scotland between 1st January 1984 and 31st December 1997. No patients had received AED therapy prior to enrolment. Patients in whom treatment with the first AED was unsuccessful entered the study. The choice of initial treatment took into account the type of seizures and epilepsy syndrome and other clinical characteristics (Brodie and French, 2000; Brodie and Kwan, 2001). After commencement of medication, patients were reviewed as described in Section 1.2.2 (Part II). Dosages were titrated according to recommended schedules (Brodie and Dichter, 1996; Dichter and Brodie, 1996) and adjusted during follow up as clinical circumstances dictated paying particular attention to efficacy and tolerability. Each patient's clinical information and progress to treatment were recorded in a prospective database (Tobias et al, 1994).

3.2.2 Definitions

Response to medication was classified as seizure-free (absence of any type of seizures or auras for at least one year); failure of treatment due to inadequate seizure control despite able to tolerate the medication (lack of efficacy) or due to adverse events (including intolerable side-effects and idiosyncratic reactions); or withdrawal of treatment for reasons unrelated to efficacy or tolerability such as concern about potential adverse events, planning a pregnancy, and a change of mind. A change of drug regimen was defined as either substitution of an existing AED or addition of a different drug. Patients who developed idiosyncratic reactions or intolerable side effects were treated with an alternative drug (substitution). When seizure control was inadequate, either substitution or an additional drug (add-on) was prescribed.

3.2.3 Statistical analysis

Patients who had to replace the first AED with a second one were analysed according to the reason for substitution. In order to address the clinical question of appropriate treatment strategy for patients who experience persistent seizures despite being able to tolerable the medication, response to substitution was compared to add-on therapy in those with inadequate seizure control on the first AED they were able to tolerate. AEDs were classified according to their putative primary mode of action (Part I; Section 3.2). Drugs that primarily act on voltage-gated sodium channels include CBZ, PHT and LTG. VGB and TGB enhance the inhibitory function of GABA. VPA, GBP and TPM are classified as having multiple mechanisms of action. The Chi-square test and Fisher's exact test were used for comparisons of categorical data. All statistical tests were two-tailed. Calculations were made using Minitab for Windows (Release 11.2) software.

3.3 Results

3.3.1 Patient demographics

Among 470 previously untreated newly diagnosed patients referred to the clinic between 1 January 1984 and 31 December 1997, treatment with the first AED was unsuccessful in 248 patients (53%), who constituted the present study cohort. One hundred and thirteen patients had inadequate seizure control on the first drug, 98 withdrew treatment due to adverse events (69 intolerable side effects, 29 idiosyncratic reactions), and 37 due to other reasons (also described in Part II; Section 1.3.6). Fifty-two percent were male. The median follow-up period was 5 years (range 2-15 years). The median age at referral was 31 years (range 9-89 years), and the median age at onset of epilepsy was 28 years (range 1-87 years).

3.3.2 Substitution vs. add-on

The percentage of successful treatment declined in each successive drug regimen (monotherapy or polytherapy), while that of patients with inadequate seizure control rose progressively (Figure 11). Among the 248 patients whose epilepsy was not controlled on the first AED, 166 received a substituted drug, 61 (37%) of whom became seizure-free on this second choice. Among the 113 patients with inadequate seizure control on the first AED, 58 opted to continue on the same drug, 24 received add-on treatment, and 31 were treated with a substituted drug, only five (16%) of whom became seizure-free (Figure 12). Eighteen patients were not able to tolerate trials of two different AEDs, five patients three AEDs, and one was not able to tolerate even the fourth choice.

When a tolerable AED was eventually identified, it was still ineffective in 56 patients, 18 of whom then received add-on treatment and four substitution, while 34 opted to continue on the same tolerated drug. Thus, among all patients with inadequate seizure control on the first AED they were able to tolerate, 42 (24 after the first drug and 18 after subsequent drugs lacked efficacy) received add-on therapy and 35 (31 after the first drug and four after subsequent drugs) received substitution. There was no significant difference (p=0.25, RR 1.5, 95% CI 0.63 to 3.71) in seizure-free rates (add-on: 11 out of 42, 26%; substitution: 6 out of 35, 17%) or incidence of adverse events necessitating withdrawal (add-on: 5 out of 42, 12%; substitution: 9 out of 35, 26%; p=0.12, RR 0.46, 95% CI 0.17 to 1.25) between the two treatment strategies (Figure 13).

3.3.3 Timing and choice of combinations

Eleven patients received add-on therapy at a later stage after the substituted drug failed to control the seizures. None of them became seizure-free, compared to a seizure-free rate of

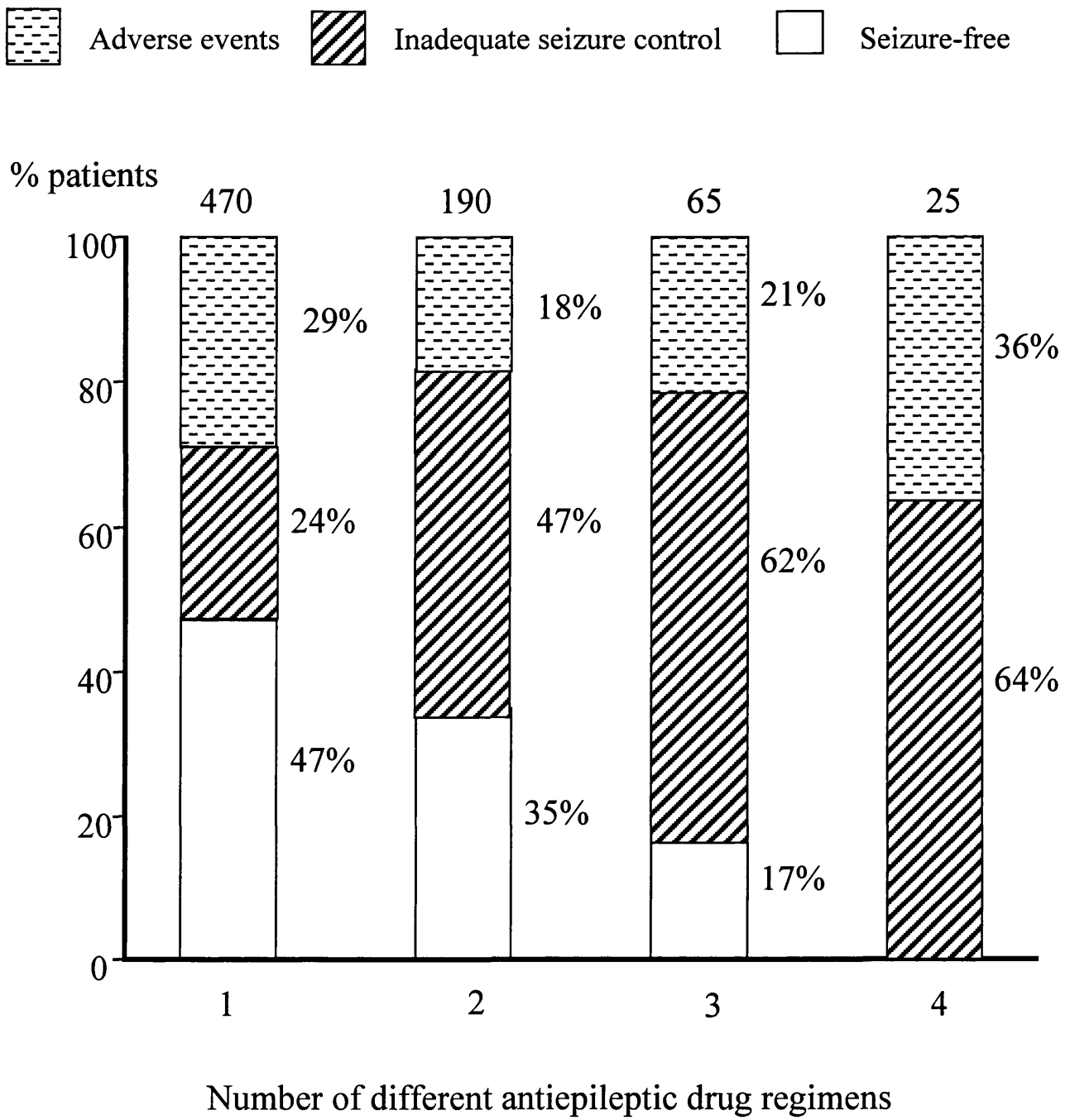


Figure 11. Response to successive antiepileptic drug regimens. Numbers of patients at risk given on top of bars.

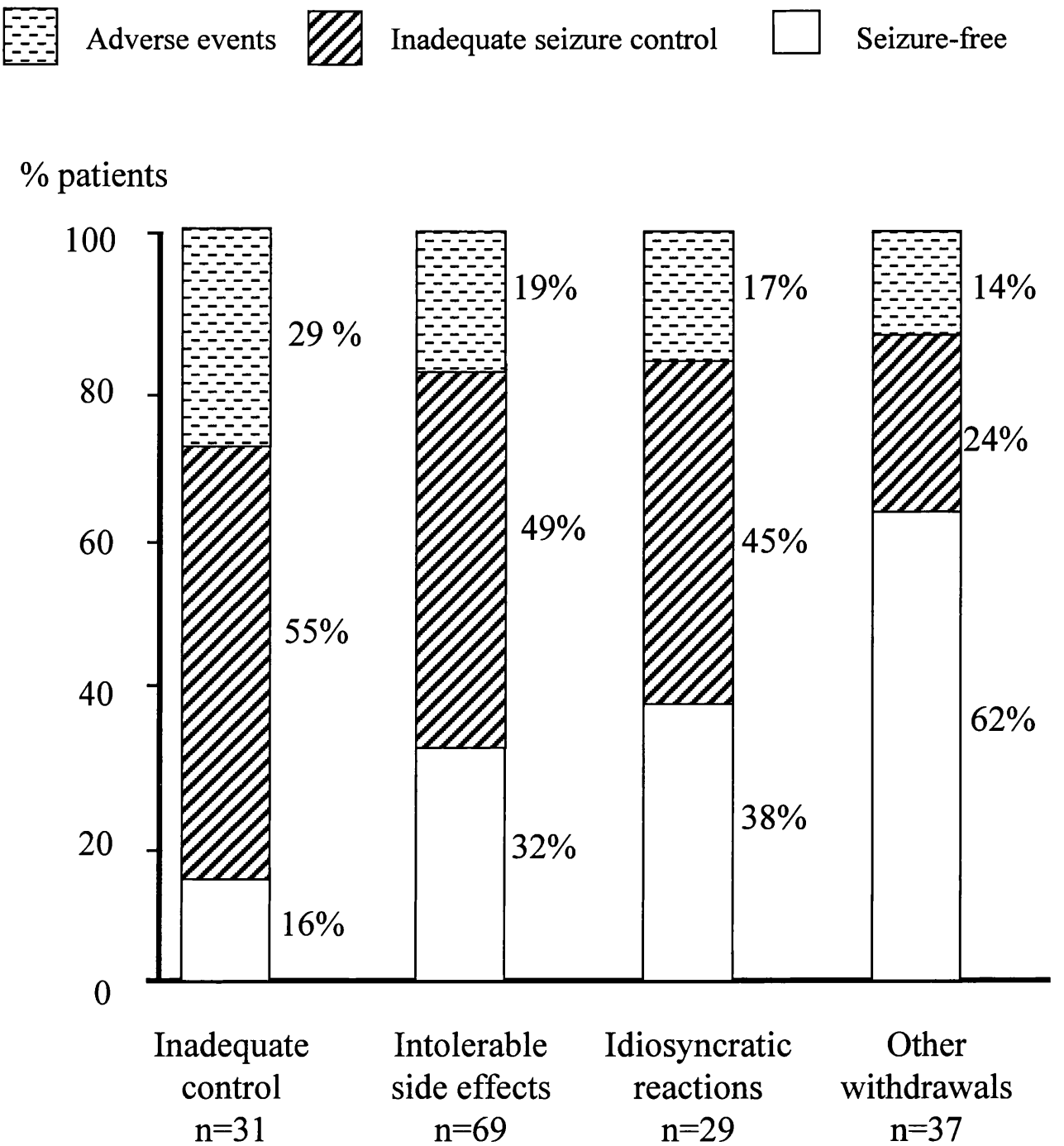


Figure 12. Response to the second antiepileptic drug according to reason for failure of the first drug. $p<0.01$.

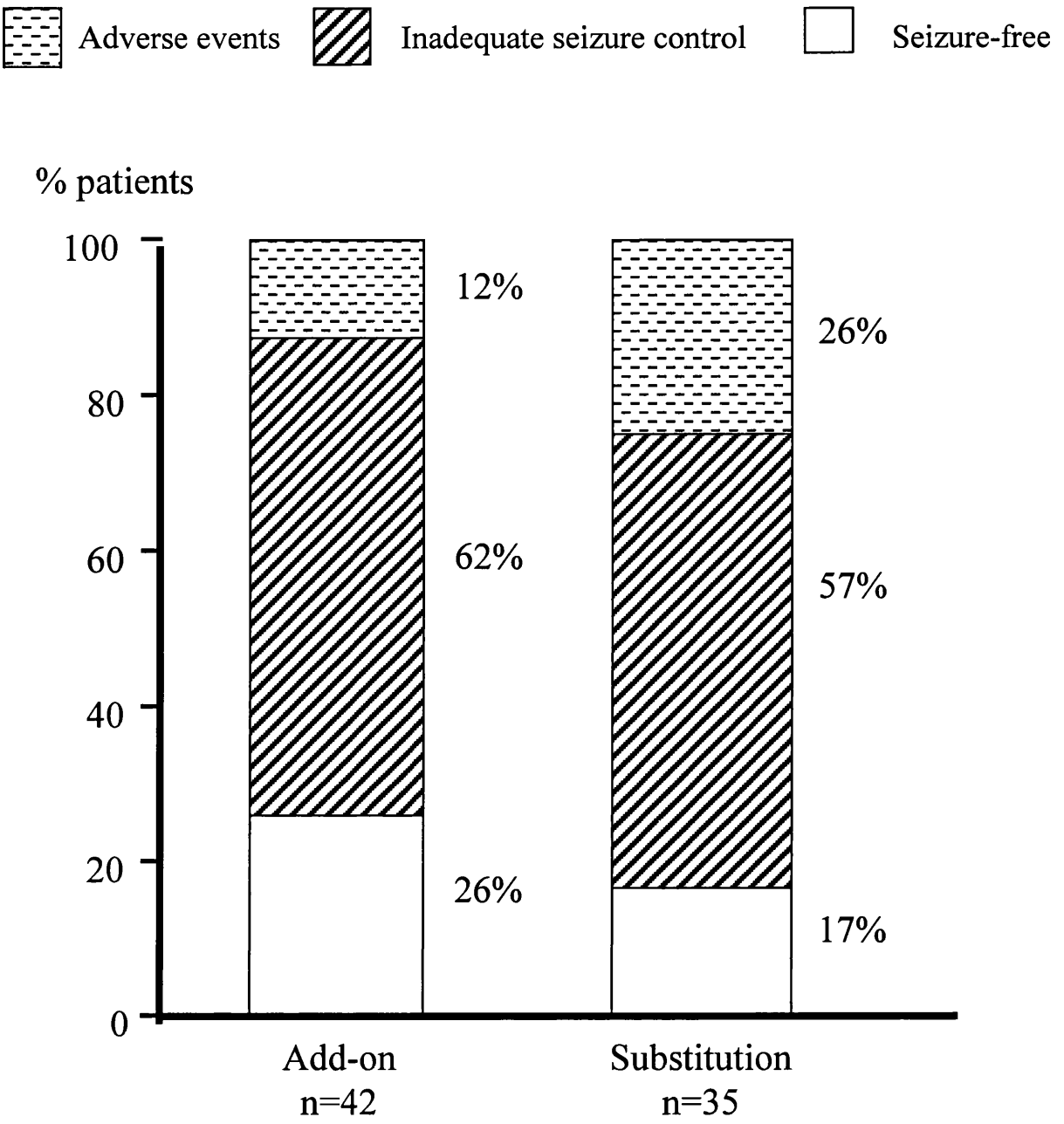


Figure 13. Response to add-on or substitution in patients with inadequate seizure control on the first well tolerated antiepileptic drug.

26% (n=42) among those who received add-on as soon as the first tolerated AED failed (p=0.05).

A variety of AED combinations were employed (Table 16). Based on the drugs' perceived primary mechanisms of action, more patients became seizure-free (Figure 14) when the combination involved a sodium channel blocker and an AED with multiple mechanisms of action (36%) compared to other combinations (7%, $p<0.05$). None of the patients who received a combination of a sodium channel blocker and a "pure" GABA-ergic agent (VGB or TGB) became seizure-free.

3.4 Discussion

Among patients with inadequate seizure control on the first well tolerated AED, those who received a substituted drug and those who received add-on had similar seizure-free rates and incidence of intolerable side effects. Based on the drugs' perceived primary mode of action, more patients became seizure-free when the combination involved a sodium channel blocker and a drug with multiple mechanisms of action compared to other combinations. Combination therapy rendered more patients seizure-free when prescribed immediately after the first drug failed due to lack of efficacy than when it was delayed until treatment with a substitution also proved unsuccessful.

The argument against add-on therapy traditionally has been its propensity to cause greater toxicity without substantial improvement in outcome (Schmidt and Gram, 1995). However, adverse events due to pharmacokinetic and pharmacodynamic interactions between AEDs can be equally problematic during the substitution phase (Brodie et al, 1997b; Brodie et al, 1999b). Only 12% of our patients given combination therapy had to withdraw treatment due to side effects, which was lower, although not significantly so,

Table 16. Combinations of antiepileptic drugs prescribed to patients receiving add-on therapy with inadequate seizure control on first well-tolerated drug.

Combinations	No. of patients
Sodium channel blocker + multiple actions	**28**
LTG + VPA	12
CBZ + GBP	6
CBZ + TPM	3
CBZ + VPA	2
LTG + TPM	3
LTG + GBP	1
PHT + VPA	1
Two sodium channel blockers	**5**
CBZ + LTG	3
CBZ + PHT	1
PHT + OXC	1
Sodium channel blocker + GABA-ergic drug	**5**
CBZ + VGB	2
LTG + VGB	2
CBZ + TGB	1
Two drugs with multiple actions	**2**
VPA + TPM	1
VPA + GBP	1
GABA-ergic + multiple actions	**1**
VGB + VPA	1
Sodium channel blocker + glutamate antagonist	**1**
PHT + REM	1
Total	**42**

CBZ=carbamazepine, GBP=gabapentin, LTG=lamotrigine, OXC=oxcarbazepine, PHT=phenytoin, REM=remacemide, TGB=tiagabine, TPM=topiramate, VGB=vigabatrin, VPA=sodium valproate, GABA=γ-aminobutyric acid

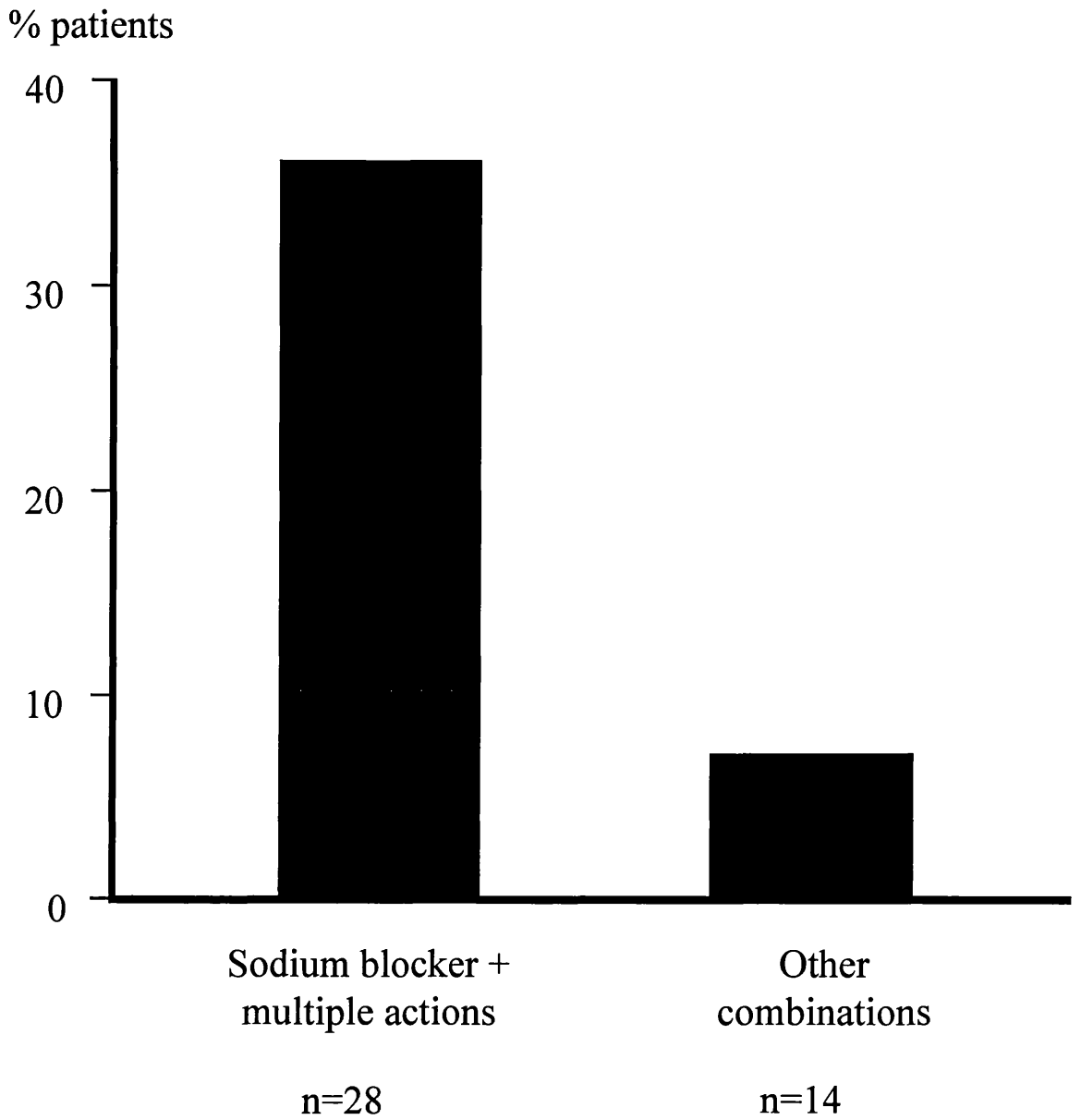


Figure 14. Response to different combinations of antiepileptic drugs according to mechanisms of action. $p<0.05$.

than those who changed to a second drug (26%). Ninety percent of the combinations employed the newer drugs, some of which are better tolerated than their older counterparts (Marson et al, 1997; Brodie, in press).

Although no significant difference in efficacy was observed between alternative monotherapy and duotherapy in our cohort, synergistic (supra-additive) effects have been demonstrated for certain specific combinations in comparative studies, notably VPA and LTG (Brodie et al, 1997b; Pisani et al, 1999). This discrepancy is likely to be due to the inclusion of a relatively small number of patients taking a large number of different combinations in the present study. Indeed, when the various combinations were grouped together according to the AEDs' primary mode of action, significantly more patients given a sodium channel blocker and a drug with multiple mechanisms became seizure-free than those treated with other combinations. As the mechanisms of action of the AEDs become better understood, it may be possible to adopt a more mechanistic approach to polytherapy (Deckers et al, 2000).

Combination therapy was more effective when prescribed immediately after the first drug failed due to lack of efficacy than when it was delayed until treatment with a substitution also proved unsuccessful. This difference in efficacy might reflect the severity of the underlying disease -- patients unresponsive to two successive AEDs might have more "drug resistant" epilepsy than those uncontrolled with a trial of just one drug. This observation again raises the question of whether pharmacoresistance is present *de novo* or evolves over time (Part I; Section 5.1). Although its clinical characteristics may be apparent early in the course of disease (Part II; Section 1), the self-perpetuating nature of seizures in some patients may account for the progressive decline in the likelihood of seizure-freedom achieved with successive treatment regimens. It is possible that some

patients have "difficult-to-control" (but still "controllable") epilepsy at the outset, which with time progresses to become "uncontrollable" or "intractable", with attendant pharmacoresistance, neuronal changes, and cognitive and psychosocial dysfunction that characterise refractory epilepsy.

Findings from this observational study may have relevance to the formulation of a more strategic approach in managing patients with newly diagnosed epilepsy. When the first AED failed due to lack of efficacy, the successful rate of an alternative monotherapy was only 16%, compared to 47% in drug-naïve patients. The chance of seizure-freedom with pharmacological treatment after failure of two consecutive AEDs due to inadequate efficacy (as opposed to poor tolerability) is slim. These patients should be assessed for epilepsy surgery if they have an operable structural abnormality. For the majority of patients not suitable for "curative" resective surgery, these preliminary observations suggest that effective rational polytherapy should be employed early since its delay, like delayed surgical intervention (Engel, 1998; 1999), might risk irreversible psychosocial consequences and reduce the chance of eliminating disabling seizures.

3.5 Conclusion

The comparisons between substitution and add-on, and between the different AED combinations inevitably suffer from confounding factors, including possible bias in patient selection and non-uniformity of drug regimen. Nevertheless, observations from this study have generated verifiable hypotheses regarding the management of epilepsy after the first AED fails. Randomised trials to evaluate the effectiveness of different AED mechanistic combinations and that of early versus late combination therapy are needed. Such a study involving newly diagnosed patients is underway in Glasgow.

4 Influence of aetiology on response to antiepileptic drug treatment

4.1 Introduction and aim

Recent advances in brain imaging, in particular MRI, have allowed better identification of the structural abnormalities underlying localisation-related epilepsy (Duncan, 1997; Spencer, 1998; Part I, Section 2.2.2). These commonly include MTS, CD, cortical gliosis, atrophy and infarction, primary neoplasm and vascular malformations such as AVM (see Part I; Sections 1.4.2 and 1.4.3). Epilepsy caused by structural abnormalities is less easy to control with AEDs than the primary generalised (idiopathic) epilepsies (Part II, Section 1). Many affected patients develop refractory partial-onset seizures which may be abolished or better controlled following resective surgery (Part I; Section 3.4). Patients with MTS-induced seizures are particularly likely to have hippocampal excision (Part I; Section 1.4.2) because of a perceived resistance to AED treatment (van Paesschen et al, 1997a; Semah et al, 1998; Briellmann et al, 1998; Kim et al, 1999). CDs are also widely thought to be associated with refractory seizures and also may be amenable to surgery (Palmini et al, 1991)

Published data are thus often concerned with surgical outcome in patients with partial-onset seizures with only a handful of studies examining the response to AED therapy. This is despite the global introduction in the last decade of nine novel AEDs for this indication (Part I; Section 3.1). This study aimed to examine the outcome in pharmacologically treated patients with localisation-related epilepsy secondary to a range of structural abnormalities.

4.2 Methods

4.2.1 Patients

The subjects consisted of adolescents and adults with localisation-related epilepsy referred to the epilepsy clinic at the Western Infirmary in Glasgow, Scotland, since 1st January 1984. At the first visit, demographic and clinical information was obtained from each patient and any witness to the seizures using a standardised structured questionnaire. Information collected included a detailed description of seizures, previous AED treatment, and risk factors for epilepsy such as febrile convulsions, head trauma, and family history of epilepsy. Seizure type and epilepsy syndromes were classified according to the guidelines of the ILAE (Part I; Sections 1.2 and 1.3). Patients in whom the clinical history or EEG results suggested localisation-related epilepsy underwent routine MRI of the brain in the 7 years prior to analysis. Only patients with localisation-related epilepsy were included in this study. They were reviewed initially every 4-6 weeks for six months and subsequently at least every 4 months for a minimum of 2 years. Compliance was monitored with the aid of on-site measurement of plasma drug concentrations at the clinic (McKee et al, 1993). Demographic and clinical data, results of investigations and response to treatment for each patient were entered systematically into an ongoing prospective database (Tobias et al, 1994).

4.2.2 Brain MRI protocol and classification of abnormalities

A standardised brain MRI protocol was employed using a 1.0-T Siemens Magnetom Scanner with a head coil. T_2-weighted turbo-spin-echo sequences were obtained to cover the whole brain in the axial plane (repetition time [TR] = 4000 ms; echo time [TE] = 120 ms; flip angle = 180°; section thickness = 5 mm; intersection gap = 0.2 mm; field of view [FOV] = 230 mm; echo train = 23). T_1-weighted MPRAGE sequences were acquired in the coronal plane (TR = 11.4 ms; TE = 4.4 ms; flip angle = 12 °; slab thickness = 250 mm;

effective thickness = 1.98 mm; FOV = 250 mm). Further 4 mm-thick high resolution T_2-weighted coronal images were acquired with TR = 4465 ms; TE = 120 ms; flip angle = 180 °; FOV = 230 mm; matrix size 300 x 512. The coronal planes were perpendicular to the longitudinal axis of the hippocampal body.

MRIs were assessed qualitatively (Part I; Section 2.2.2) and all films were reviewed blind by the same radiologist with special interest in neuroradiology to ensure the veracity of the original report. Abnormalities were classified as CD (abnormalities of gyration, heterotopia, tuberous sclerosis, focal cortical dysplasia, microdysgenesis, dysembryoplastic neuroepithelial tumour, megalencephaly / hemimegalencephaly); MTS; cortical gliosis (post-traumatic brain injury); cerebral infarction; tumours (glioma, meningioma); AVM; and cerebral atrophy (Part I; Section 1.4.3). Patients with dual pathology (MTS and another structural lesion) and those with surgically resectable tumours were excluded. The latter were referred to another centre for consideration of epilepsy surgery.

4.2.3 Statistical analysis

Patients were divided into two groups according to whether they were seizure-free or uncontrolled. Seizure-freedom was defined as the absence of any type of seizures or auras for a minimum of one continuous year. Seizure control was assessed at the time of the patient's last clinic visit. The Chi-square test was used for comparisons of categorical data. All statistical tests were two-tailed. Calculations were computed using Minitab for Windows (version 11.21) software.

4.3 Results

4.3.1 Patient demographics

Five hundred and fifty patients (50% male, 50% female) with localisation-related epilepsy entered the study. Of these, 165 (30%) were taking AED treatment at time of referral. The median age at onset of epilepsy was 21 years (range <1-92 years). The median age at referral was 30 years (range 12-93 years). The median follow-up period was five years (range 2-15 years).

4.3.2 Brain MRI findings

Structural abnormalities were identified on brain MRI in 361 patients (66%), who were classified as having symptomatic epilepsy. Eighty-one patients (15%) had cortical gliosis, 73 (13%) MTS, 63 (12%) CD, 49 (9%) cerebral atrophy, 46 (8%) cerebral infarction, 35 (6%) primary tumour, and 14 (3%) AVM. Brain MRI was normal in the remaining 189 patients (34%), who were classified as having cryptogenic epilepsy. No patient was classified as having idiopathic localisation-related epilepsy in this cohort of adolescent and adult patients.

4.3.3 Treatment outcome

Overall, 312 (57%) patients were seizure-free at the time of analysis. There was no difference in outcome between patients with symptomatic or cryptogenic epilepsy (56% versus 58% seizure-free, respectively; RR 0.96, 95% confidence interval (CI) 0.8 to 1.1). Patients with MTS (n=73, 42% seizure-free) were less likely to be seizure-free (Figure 15; $p<0.01$, relative risk 1.4, 95% CI 1.1 to 1.8), than those with other pathologies (AVM: n=14, 78%; cerebral infarction: n=46, 67%; primary neoplasm: n=35, 63%; cortical gliosis: n=81, 57%; cerebral atrophy: n=49, 55% and CD: n=63, 54%). There was no statistical

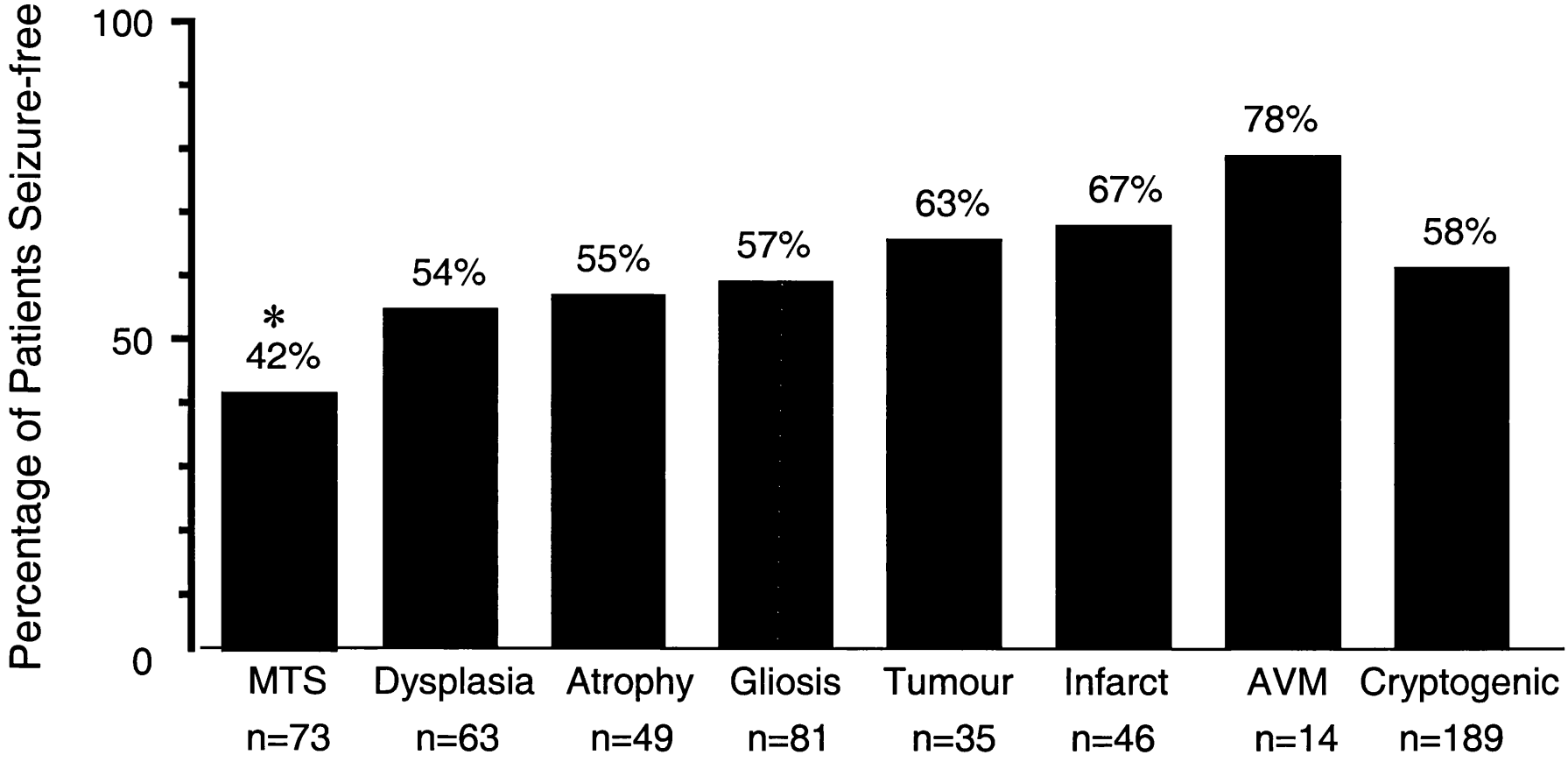


Figure 15. Seizure control in different patient groups. $p<0.01$ compared with other abnormalities.
MTS = mesial temporal sclerosis, AVM = arteriovenous malformation.

difference in outcome among patients with other lesions. Of the 63 patients with CD, 34 (54%) were seizure-free. Thirty-six categories of dysplastic lesion were identified on brain MRI, the commonest being right temporal heterotopia (n=7) and bilateral periventricular tubers (n=6). No specific dysplastic lesion was associated with a particularly poor prognosis.

4.3.4 Antiepileptic drug regimens

Of the 312 seizure-free patients, 187 patients (60%) were receiving treatment with a single AED (Table 17). Eighty-three patients (27%) required two AEDs, while 15 (4.7%) were controlled with three drugs, and one took four. Thirty-four patients elected not to take an AED, 26 having never been treated and eight having discontinued therapy due to intolerable side-effects. Of these 34 individuals, 26 experienced only sporadic seizures with none in the previous year, while the remaining eight had more frequent seizures. Among the seizure-free patients, 48% of patients with MTS required more than one AED ($p<0.05$, relative risk 1.6, 95% CI 1.1 to 2.4) compared to 35% of those with other aetiologies (20% with CD, 23% with primary tumour, 27% with AVM, 29% with infarction, 30% with gliosis, 44% with atrophy and 31% with cryptogenic epilepsy). Patients with uncontrolled epilepsy also took a range of AEDs, varying from none to four (Table 18).

4.3.5 Family history and history of febrile convulsion

Family history of epilepsy (first or second degree) was more common (Table 19; $p=0.02$) in patients with MTS, CD and cryptogenic epilepsy (all 25%) and AVMs (28%) than in those with atrophy (20%), infarction (15%), gliosis (9%) and neoplasia (6%). The presence of a family history tended to convey a better prognosis in the MTS patients (present: n=18,

Table 17. Seizure-free patients on no treatment and on different antiepileptic drug (AED) regimens.

Aetiology	Patients n (%)	No AED n (%)	1 AED n (%)	2 AEDs n (%)	3 AEDs n (%)	4 AEDs n (%)
MTS	31 (100)	7 (23)	9 (29)	12 (39)	2 (6)	1 (3)
CD	34 (100)	5 (15)	22 (65)	6 (17)	1 (3)	0 (0)
Atrophy	27 (100)	1 (4)	14 (52)	11 (40)	1 (4)	0 (0)
Gliosis	46 (100)	3 (7)	29 (63)	12 (26)	2 (4)	0 (0)
Tumour	22 (100)	1 (5)	16 (72)	4 (18)	1 (5)	0 (0)
Infarction	31 (100)	1 (3)	21 (68)	7 (22)	2 (7)	0 (0)
AVM	11 (100)	1 (9)	7 (64)	2 (18)	1 (9)	0 (0)
Cryptogenic	110 (100)	7 (6)	69 (63)	29 (27)	5 (4)	0 (0)
Total	312 (100)	26 (8)	187 (60)	83 (27)	15 (4.7)	1 (0.3)

MTS = mesial temporal sclerosis; CD = cortical dysgenesis; AVM = arteriovenous malformation

Table 18. Patients uncontrolled on no treatment and on different antiepileptic drug (AED) regimens.

Aetiology	Patients n (%)	No AED n (%)	1 AED n (%)	2 AEDs n (%)	3 AEDs n (%)	4 AEDs n (%)
MTS	42 (100)	1 (2)	7 (17)	24 (57)	10 (24)	0 (0)
CD	29 (100)	2 (7)	10 (34)	13 (45)	3 (10)	1(4)
Atrophy	22 (100)	1 (4)	9 (41)	8 (37)	3 (14)	1 (4)
Gliosis	35 (100)	1 (3)	15 (43)	11 (31)	8 (23)	0 (0)
Tumour	13 (100)	0 (0)	2 (15)	7 (54)	4 (31)	0 (0)
Infarction	15 (100)	0 (0)	7 (47)	6 (40)	2 (13)	0 (0)
AVM	3 (100)	0 (0)	1 (33)	2 (67)	0 (0)	0 (0)
Cryptogenic	79 (100)	3 (4)	29 (36)	41 (52)	6 (8)	0 (0)
Total	238 (100)	8 (3)	80 (34)	112 (47)	36 (15)	2 (1)

MTS=mesial temporal sclerosis; CD=cortical dysplasia; AVM=arteriovenous malformation

Table 19. Family history of epilepsy and history of febrile convulsions in different patient groups.

	MTS	CD	Atrophy	Gliosis	Tumour	Infarction	AVM	Normal MRI
No. of patients	73	63	49	81	35	46	14	189
Family history (%)	18* (25)	16* (25)	10 (20)	8 (9)	2 (6)	7 (15)	4* (28)	48* (25)
Febrile convulsion (%)	27+ (37)	7 (11)	8 (16)	3 (4)	0 (0)	2 (4)	0 (0)	23 (12)

MTS=mesial temporal sclerosis, CD=cortical dysplasia, AVM=arteriovenous malformation; MRI=magnetic resonance imaging

* $p<0.02$; + $p<0.001$ compared with other patient groups.

61% seizure-free; absent: n=55, 36% seizure-free; p=0.065, RR 1.6, 95% CI 1.0 to 2.8). MTS patients were more likely to have had febrile convulsions (Table 19) than those in the other groups (p<0.001, relative risk 4.1, 95% CI 2.7 to 6.2), although this did not appear to affect outcome (present: n=27, 41% seizure-free; absent: n=46, 43% seizure-free).

4.4 Discussion

Over half of the patients with pharmacologically managed localisation-related epilepsy in this study become seizure-free. The majority were taking monotherapy. MTS patients were significantly more likely to be refractory and to require more than one AED to attain seizure-freedom. Within this pathological grouping, however, there was substantial variation in seizure control, ranging from seizure-freedom on no AEDs to refractory on four. This phenomenon was observed in patients with other diagnoses. Complete seizure control was achieved in the majority of patients with CD. No difference in outcome was found between patients with symptomatic or cryptogenic epilepsy. The latter had similar rates to patients with CD for seizure-freedom, and epilepsy risk factors. A family history of epilepsy was more common in patients with MTS, CD, cryptogenic epilepsy and AVM. This characteristic seemed to convey a better prognosis for patients with MTS. A history of febrile convulsions was statistically more prevalent in MTS patients compared to those with other pathologies.

Consistent with other reports (Part I; Section 1.4.2), patients with MTS had the poorest prognosis. There are few data, however, with which these outcomes can be compared. Semah and colleagues (1998) studied seizure frequency over one year in a cohort of 1369 out-patients with localisation-related epilepsy. As in the present study, patients with infarcts, vascular malformations and tumours had the best prognosis with 54%, 50% and 46% being seizure-free respectively. Their outcomes for MTS patients were poorer with

only 11% being fully controlled. Only 8% of the cohort were newly diagnosed, however, and the remaining pharmacoresistant patients contributed to the low seizure-freedom rates. Van Paesschen and co-workers (1997) examined for one year 63 out-patients with seizures who underwent brain MRI. Of these, all six with MTS remained refractory despite AED treatment, unlike 57 with other pathology, 30 of whom became seizure-free. Kim and colleagues (1999) reported that 25% of 104 out-patients taking AED treatment for MTS remained seizure-free during 2 years' follow-up. Again, the majority were previously taking AEDs at time of referral with only 15% being newly diagnosed. Briellmann and colleagues (1998) also published a report on 47 patients with MTS being assessed for surgery. Not surprisingly, none was seizure-free.

The reason of the particular pharmacoresistant nature of MTS is unclear (Part I; Section 5.2). Over-expression of P-glycoprotein to limit AED access to the seizure focus has been suggested (Part I; Section 5.2.2). The potential role of P-glycoprotein in epilepsy will be discussed in detail in Part III.

Over half of the patients with CD in the present study attained complete seizure control. These patients fared much better than those studied by Semah and colleagues (1998) who observed that only 24% of patients with this diagnosis were seizure-free. Palmini and co-workers (1991) examined 30 patients with CD-related refractory seizures. All but five had never experienced remission of their seizures. This study may have selected out a particularly pharmacoresistant population, however, since these patients were being considered for epilepsy surgery. The variability in outcomes within CD patients may reflect the wide range of lesions which comprise the condition (Kazee et al, 1991; Armstrong, 1996; Raymond et al, 1995; 1996).

While patients with MTS and CD in this report had a more promising outlook than those previously studied, there was substantial heterogeneity among this group with some patients in remission off treatment while others continued to have seizures despite taking up to 4 AEDs. Murakami and colleagues (1996) observed this variability in children with MTS. The phenomenon may be a reflection of the natural history of the heterogeneous conditions, although this is difficult to explore given the low incidence and the fact that the course may be altered by AED therapy and/or surgery in most patients with seizures. The reporting at autopsy of MTS among non-epileptic subjects substantiates this theory (Crome, 1955; Crystal et al, 1993; Dickson et al, 1994).

A family history of epilepsy was present in 25% patients with MTS. Interestingly, this seemed to convey a better prognosis, suggesting perhaps a hereditary component to the disease process in some patients (Fernandez et al, 1998). Such an association has been noted in patients who underwent temporal resection, although they had a variety of pathologies (Abou-Khalil et al, 1993). Febrile convulsions occurred in 37% patients with MTS, a higher figure than in all other groups. This is a well-recognised relationship (Fernandez et al, 1998). A history of febrile convulsions did not affect outcome in the present study. In previous studies, this has been associated with poorer response to AED treatment (Kim et al, 1999), but better outcome after temporal resection (Abou-Khalil et al, 1993). It has been postulated that a pre-existing hippocampal lesion may predispose some individuals to febrile convulsions, MTS and a tendency towards refractory epilepsy, rather than febrile convulsions being the causative factor (Fernandez et al, 1998; Fisher et al, 1998; Hamati-Haddad and Abou-Khalil, 1998).

Patients with CD and AVM had a higher prevalence of family history of epilepsy than those with infarcts, gliosis and neoplasia. CD may be the result of familial disorders such

as tuberous sclerosis or neurocutaneous syndromes (Shorvon, 1997). MTS with duplication / dispersion of the dentate fascia may be a form of CD (Raymond et al, 1994). The association of family history in patients with AVM may be explained by previous MRI findings that up to 75% sporadic cases of cavernous angiomas are familial (Labauge et al, 1998).

Characteristics of patients with normal brain imaging were similar to those with CD, in terms of percentages of patients seizure-free (58% and 54% respectively), those with a family history of epilepsy (25% each), and those with a history of febrile convulsions (12% and 11% respectively). This lends credence to the suggestion that some patients with cryptogenic epilepsy may have cortical microdysplasia. Microdysgenesis can increasingly be identified by sophisticated neuroimaging techniques (Sisodiya et al, 1996).

4.5 Conclusion

In conclusion, outcome in patients pharmacologically treated for symptomatic or cryptogenic localisation-related epilepsy was highly variable. Whilst MTS was associated with the poorest prognosis, many more patients with the diagnosis were seizure-free than in previous studies. Encouraging results were also obtained for patients with CD. Although many patients with MTS and some with CD will benefit from resective surgery, a considerable number will be controlled on AED therapy. Multicentre prospective studies are required to assess the clinical course in pharmacologically treated patients with newly diagnosed localisation-related epilepsy as a consequence of these pathologies.

5 Glutamic acid decarboxylase autoantibodies in epilepsy

5.1 Introduction and aim

GABA, the major inhibitory neurotransmitter in mammalian brain, is formed exclusively from L-glutamic acid via an irreversible reaction catalysed by GAD (Fariello et al, 1991; Part I; Section 3.2.4). Apart from GABA-ergic neurons in the central and peripheral nervous systems, GAD is present in pancreatic β-cells, epithelial cells of the fallopian tube, and within the spermatozoa of the testes (Folli, 1998; Erdo and Wolff, 1990). GAD is a dominant autoantigen in insulin dependent diabetes mellitus (IDDM) and stiff man syndrome (SMS), a rare neurological disorder characterised by progressive and fluctuating muscle rigidity and painful spasms (Ellis and Atkinson, 1996; Atkinson, 2000). In IDDM, immune response to GAD and other islet-cell antigens is thought to be involved in the destruction of insulin-secreting cells (Lohmann et al, 2000) while GAD autoantibodies have been suggested to inhibit GABA synthesis in SMS (Dinkel et al, 1998).

There have been recent case reports of elevated GAD autoantibodies in patients with refractory partial seizures (Part I; Section 5.2.2). Impairment of GABA function is recognised to provoke seizures while glutamate is a pro-convulsant (Meldrum, 1995). Many AEDs exert their anticonvulsant effect by facilitating GABA-ergic activities (Table 4). Based on such theoretical consideration and anecdotal case reports, a causal relationship between GAD autoantibodies and refractory epilepsy has been postulated and immunotherapy has been advocated as treatment (Giometto et al, 1998). Whether GAD autoantibodies are associated with seizure disorders in general or with specific epilepsy syndromes, and perhaps more importantly, with refractoriness, has not been studied.

To explore the hypothesis that elevated GAD antoantibodies may be associated with therapy resistance, a pilot study was conducted to compare titres between patients with

controlled and uncontrolled epilepsy. To maximise the applicability of the results to everyday clinical practice, common epilepsy types and aetiologies of both idiopathic and symptomatic nature were chosen.

5.2 Methods

5.2.1 Patients

One hundred and five patients (44% male) with a diagnosis of juvenile myoclonic epilepsy (an idiopathic syndrome), or with partial-onset seizures as a result of mesial temporal sclerosis, cortical dysplasia, or cortical gliosis were enrolled. Thirty one patients had been seizure-free for at least one year while the epilepsy had been refractory to AED treatment in the remaining 74 patients (Table 20). All were attending the Epilepsy Unit in Glasgow, Scotland. Median age was 37 years (range 17 to 71 years) and median duration of epilepsy was 13 years (range 2 to 59). None of the patients had a history of IDDM or SMS. The study was approved by the Western Ethics Committee and all patients gave written informed consent to their participation.

5.2.2 Measurement of serum GAD autoantibodies

GAD autoantibodies were measured in the serum by radioimmunoassay, which has been established as a sensitive and specific detection technique (Schranz and Lernmark, 1998). Aliquots of 100μl of standards (human serum containing GAD antibodies supplied at different dilutions expressed in arbitrary units, U/mL) and serum samples were incubated with 200μl of I^{125}-labelled GAD antibodies complexed to GAD in tubes coated with GAD antibodies for 24 hours at room temperature (BRHAMS Diagnostic GmbH, Berlin, Germany). Extra tracer was washed off and radioactivity of each tube was measured in a gamma scintillation counter for 1 minute. The amount of radioactivity was inversely

Table 20. Median glutamic acid decarboxylase autoantibody titres (confidence intervals) in patients with controlled and uncontrolled epilepsy.

	Controlled		Uncontrolled	
Syndrome	No.	Titre (95% CI)	No.	Titre (95% CI)
Mesial temporal sclerosis	9	0.48 (0.22-0.91)	31	0.56 (0.33-0.86)
Cortical gliosis	7	0.46 (0.08-1.12)	17	0.46 (0.16-0.65)
Cortical dysgenesis	7	0.57 (0.25-0.77)	15	0.63 (0.23-0.84)
Juvenile myoclonic epilepsy	8	0.68 (0.26-1.50)	11	0.40 (0.16-0.96)
Total	31	0.48 (0.36-0.78)	74	0.50 (0.37-0.65)

proportional to the concentration of autoantibodies in the patient sample. The results were interpolated in the standard curve. All samples were tested in duplicate and the mean taken for presentation.

5.2.3 Other tests

Random blood glucose was measured in all patients to screen for undiagnosed diabetes mellitus. The sera of patients with elevated GAD autoantibody titres were tested for other autoantibodies, including pancreatic islet cell antibody, antinuclear antibody, extranuclear antibodies, anti-gastric parietal cell antibody, intrinsic factor antibody, anti-thyroid antibodies, and rheumatoid factor.

5.2.4 Statistical analysis

The Mann-Whitney test was used for comparisons of non-parametric continuous data. Correlation was employed to examine the possible association between GAD autoantibody titre and duration of epilepsy or seizure frequency. Statistical calculations were performed with use of Minitab for Windows software (version 11.21).

5.3 Results

There was no significant difference in serum GAD autoantibody titres between patients who were seizure-free and those with uncontrolled epilepsy, either analysing the syndromes in combination (Figure 16) or individually (Table 20). There was no significant association between the titre of GAD autoantibody and duration of epilepsy or seizure frequency. However, four female patients with uncontrolled epilepsy had GAD autoantibody levels at least three times above the highest titre measured in the seizure-free cohort (Table 21). Two of the four patients had mesial temporal sclerosis and one cortical dysplasia. The highest titre by far (170.4 U/ml) was found in the fourth patient (patient 1

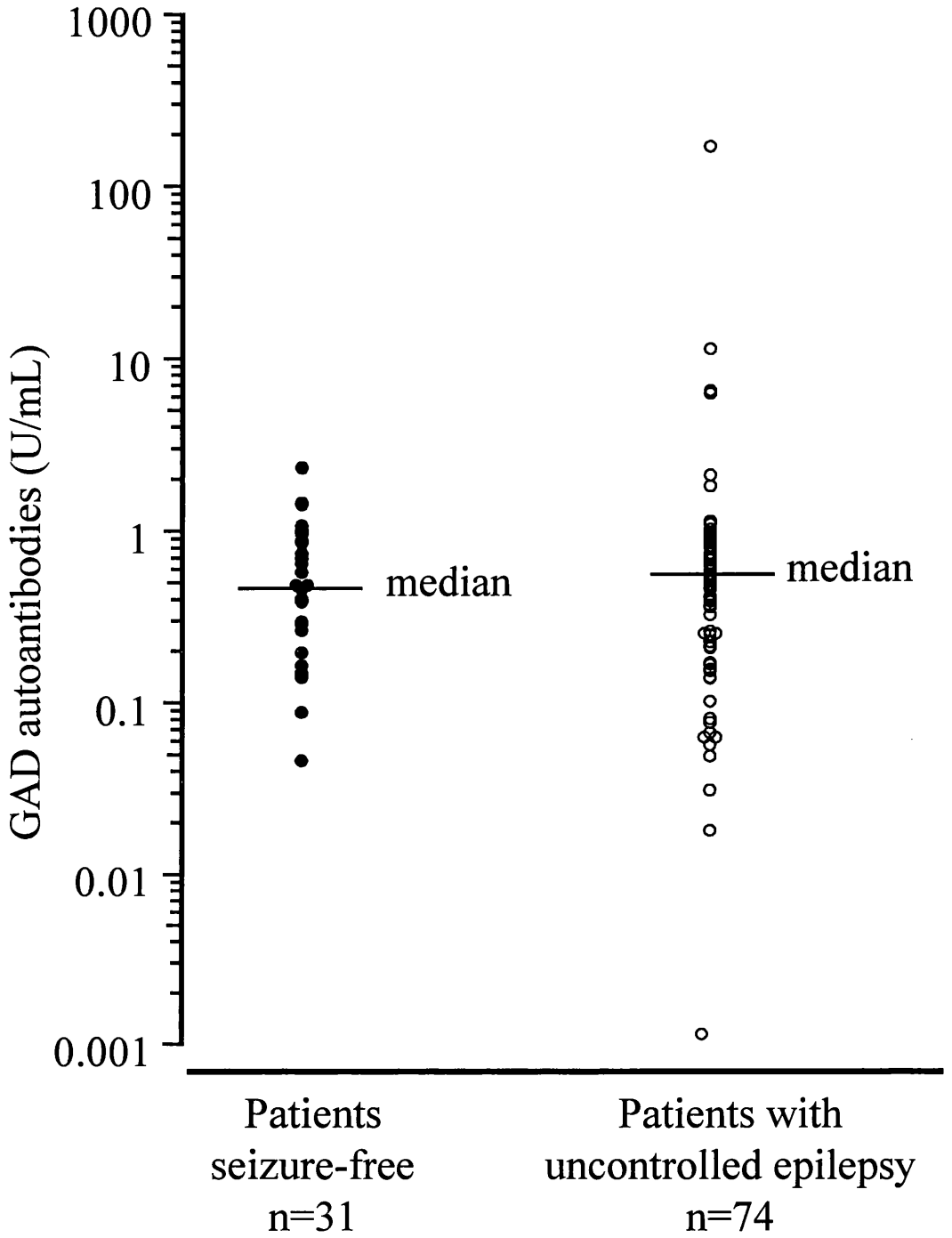


Figure 16. Glutamic acid decarboxylase (GAD) antoantibody titres in patientswith controlled and uncontrolled epilepsy.

on Table 21) who had juvenile myoclonic epilepsy. She also tested positive for pancreatic islet cell antibody and antinuclear antibody (1 in 640). Pancreatic islet cell antibodies were detected in two other patients (patients 3 and 4 on Table 21). None had other systemic or organ-specific autoantibodies, or a family history of IDDM or autoimmune diseases. Random blood glucose levels were normal in all four. All had been treated unsuccessfully by a variety of AEDs with different mechanisms of action (Table 4), including facilitation of GABA-ergic activity.

5.4 Discussion

A causal relationship between GAD autoantibodies and epilepsy has been postulated based on anecdotal case reports, and immunotherapy, such as corticosteroids and intravenous immunoglobulins, has been advocated as treatment (Giometto et al, 1998). However, in these patients, additional features were present such as SMS (Solimena et al, 1988), diabetes mellitus (Saiz et al, 1997), impaired glucose tolerance (Nemni et al, 1994), or acute encephalitis (Giometto et al, 1998), all of which might explain the presence of GAD autoantibodies.

No difference in GAD autoantibody titres between seizure-free patients and those with refractory epilepsy was detected in this pilot study, or any relationship between GAD autoantibody titre and seizure frequency or duration. Four female patients with refractory epilepsy did appear to have higher antibody levels compared with the rest of the cohort. However, three of them also harboured pancreatic islet cell antibodies, which can be found in association with IDDM (Schranz and Lernmark, 1998), suggesting co-existing autoimmune damage to the pancreas and a possible pancreatic source of the GAD

Table 21. Clinical characteristics of four female patients with elevated glutamic acid decarboxylase (GAD) autoantibody titres.

Patient	Age (years)	Underlying aetiology	No. of seizures per month	Current Treatment	Previous Treatment	GAD antibody titre (U/mL)	Pancreatic islet cell antibodies
1	27	JME	1	VPA*	--	170.4	Strongly positive
2	33	MTS	8-12	VPA*, LTG, TPM*	--	11.3	Negative
3	41	MTS	2-4	VPA*, LTG	PB*, TPM*, CLB*	6.5	Borderline
4	20	CD	1	CBZ	VPA*, GBP*	6.3	Positive

JME, juvenile myoclonic epilepsy; MTS, mesial temporal sclerosis; CD, cortical dysplasia; CBZ, carbamazepine; CLB, clobazam; GBP, gabapentin; LTG, lamotrigine; PB, phenobarbital; TPM, topiramate; VPA, sodium valproate.

* Antiepileptic drugs known to facilitate GABA-ergic activity (Table 4).

antoantibodies. There were no other shared clinical characteristics among these patients. Theoretically, they should be more responsive to AEDs that enhance GABA function, but all had failed treatment with GABAergic agents.

There has been recent renewed interest in the potential role of the immune system in the pathogenesis of epilepsy. Antibodies to glutamate receptor subtype GluR3 have been found in patients with Rasmussen's encephalitis, but this is not always so and the condition often does not respond to immunotherapy (Dulac, 1996). Anti-GM1 antibodies were detected in 4 out of 64 patients with epilepsy in one study (Bartolomei et al, 1996) but whether the antibodies affect treatment outcome has not been examined. Likewise, the clinical significance of raised GAD autoantibodies in epilepsy remains to be determined. A recent Finnish study detected GAD autoantibodies in 8 out of 100 patients, but the two with highest levels had either a clinical history or serological evidence of autoimmune disease (Peltola et al, 2000). Finding of elevated GAD autoantibody titre in a patient with drug-resistant epilepsy should, therefore, prompt a search for other autoantibodies, particularly those against the pancreas.

5.5 Conclusion

Although results from this pilot study do not suggest an association between elevated GAD autoantibodies and pharmacoresistance in common epilepsy types, studies in larger patient populations are required to confirm this. It is possible that their presence or influence on outcome is syndrome-specific, and was, therefore, not detected in the mixed patient sample

studied. Any potential therapeutic role of immunotherapy needs to be evaluated in a double-blind, placebo-controlled trial.

6 General discussion and conclusion

Epidemiology can provide understanding about the incidence, prevalence, associated mortality and potential risk factors for a given condition. These aspects have been vigorously studied in epilepsy patients across continents in recent years (Part I; Section 1). However, it is the understanding of the natural history that potentially can have a direct impact on planning health services for people with epilepsy. Formulation of a more rational and strategic approach to the management of epilepsy thus formed the underlying objective of the series of prospective observational studies involving newly diagnosed patients described in this part of the thesis.

6.1 Methodological factors concerning study design and statistics

The series of outcome studies described in Part II, Sections 1-4, undoubtedly suffer from a number of methodological disadvantages, partly resulting from the setting in which they were carried out. The studies were conducted in a single centre. In order to maximise the study population and to document the long-term outcome of newly diagnosed patients, the study period stretched some 15 years. Such a long duration may be a weakness in itself in view of the many momentus changes in the management of epilepsy over this period of time, including classification, imaging technology and treatment.

To compensate for these possible confounding factors, the classification for each patient took into account all accumulated clinical and investigational information over the years and reflected the final classification at the follow up. Although many new AEDs have entered the market over the study period, they are still being prescribed to a minority of patients and there

is no strong evidence that they have made a substantial impact on the prognosis of epilepsy, at least in the fashion they are presently utilised. In addition, the studies aimed to seek a "generic" understanding of patients' reponse to AED treatment. Specific drugs were not compared except in an exploratory manner for the first AED prescribed in Part II, Section 2.

Conducting clinical studies in a single centre also risks the possibility of patient selection bias imposed by the characteristics of the centre and the setup of the health care service in which it operates. Apart from the Epilepsy Unit at the Western Infirmary, the other major epilepsy service in Glasgow is situated at a separate hospital specialising in the management of neurological diseases (the Institute of Neurological Sciences). It is possible that general practitioners might preferentially refer patients with associated neurological disorders to the latter. Since such patients in general have a poorer prognosis (Annegers et al, 1979), results from the Epilepsy Unit described in this thesis might have over-estimated the seizure-free rate.

Perhaps the most important limitation of the results relate to the nature of the studies. In the strictest sense, these studies were not "prospective" since it was not possible to define the exact parameters and variables to be analysed some 15 years ago. On the other hand, data were collected "longitudinally" and patients were unselected apart from the criteria that they were newly diagnosed or not on AED treatment at inclusion. This should have minimised the amount of missing data seen in the more typically "retrospective" or "case review" studies.

The parameters and statistical techniques employed to assess outcome in these studies also need discussion. Outcome definitions used in the literature are heterogeneous. Parameters

such as number of seizures, seizure frequency, time to recurrence of seizures, time to remission, attainment of remission for a certain length ("remission ever" or "cumulative remission"), and length of "terminal remission" have all been used. The last two have been the favoured measurements in the more recent studies (Shafer et al, 1988; Cockerell et al, 1995b). Measuring "remission ever" may not be relevant if the epilepsy subsequently becomes resistant to treatment after relapse. Terminal remission was chosen as the outcome measure in the studies described in this thesis. It has the practical advange of easy applicability and is probably most clinical relevant since it has a direct bearing on a patient's present quality of life. Its main disadvantage is perhaps over-estimation of poor outcome since occurrence of only one seizure in the last year of follow-up would result in classification into the poor outcome group. A combination of terminal remission and longest cumulative remission period would probably be the best measurement.

Previous large-scale outcome studies (Shafer et al, 1988; Cockerell et al, 1995b) have tended to use the acturial, life-table approach to assess the probability of remission and compare risk factors, although the more straight-forward logistic regression analysis have also been used (Arts et al, 1999). The former has the clear advantage of describing outcome in relation to time, whereas the latter can only analyse predictors of outcome variables in a binary fashion. The main aim of the outcome studies described in this thesis was to document the course of epilepsy in relation to drug treatment instead of the traditional time factor (see Part I; Section 4.2.2). A "suvival curve" of seizure-freedom in relation to successive drug regimen was constructed for this purpose (Figure 7). Another important aim was to examine the predictive value of the response to the first AED for final outcome, which has not been attempted in

previous studies. In view of the exploratory nature of this analysis, relatively simple binary analysis was adopted to generate results that would guide the design of future studies. It is acknowledged that in hindsight, the more sophisticated acturial approach would probably produce additional useful information, and should certainly be employed in more definitive prospective studies in the future (see Overall Discussion and Conclusion).

6.2 Newly diagnosed epilepsy – a continuum or two populations?

The past decade has witnessed unprecedented advances in the understanding of the pathophysiology of seizures and epilepsy, diagnostic and investigational technologies (Part I; Section 2.2), coupled with a more realistic assessment of the long-term prognosis of the epilepsies (Part I; Section 4.2). Since effective treatment for epilepsy has been available for nearly a century, modern outcome studies (at least in developed countries where treatment is widely available) reflect the natural history of epilepsy in response to treatment. Yet in spite of the considerable number of such studies and their consistent finding of a remission rate of 65-80% (Table 7), there is a paucity of documentation concerning the chronological progress from the point of diagnosis (or initiation of treatment) to the state of seizure-freedom or "refractoriness". In other words, what is the likelihood of success or failure of each successive treatment regimen? The importance of such knowledge might not have been apparent when there were only limited therapeutic options of similar efficacy. However, this is no longer the case in epilepsy where there has been an exponential increase in the number of AEDs (Part I; Section 3.1) and a dramatic improvement in surgical outcome of selected patient groups since the 1980s (Part I; Section 3.4). For the (still) minority with surgically correctable lesions, a better understanding of the natural history of epilepsy in response to

AED treatment is required to aid deciding the timing of operation. For the majority "non-surgical candidates", such knowledge can provide a framework to guide practical AED use, in terms of when, how and what monotherapy or polytherapy should be employed.

Central to this management template are the two seemingly antagonistic concepts concerning the development of "refractoriness" -- is it a *de novo*, "predetermined", inevitable state, or an evolving, and thus potentially preventable process (Part I; Section 5.2)? Is it possible that they are both "correct"? Many hold that the outcome of most epilepsies is determined primarily by aetiology and syndromic classification (Perucca et al, 2000). This view is supported by findings of the study on newly diagnosed patients described in Part II; Section 1, that subsequent "refractoriness" may be predicted by clinical characteristics present early in the course of the disease. Such parameters included a structural cerebral abnormality, 20 or more seizures before starting treatment, and, most importantly, the reason for failure on the first AED. Only 11% of patients who had inadequate seizure control (as opposed to poor tolerability) on a sizeable dose of the first AED later became seizure-free (Figure 6).

When treatment outcome on the first ever AED was examined more closely in Part II; Section 2, a similar response pattern to the most commonly prescribed drugs emerged. More than 90% of the responding patients to the first AED required a moderate dose (CBZ up to 800mg, VPA up to 1500mg, LTG up to 300mg) with few more becoming seizure-free at higher dosage. Interestingly, poor tolerability largely occurred at doses lower than needed for good efficacy, and occurred more commonly in patients with symptomatic/cryptogenic epilepsies than idiopathic epilepsies. These results push the likely recognition of subsequent

"refractoriness" even further, to the point when a moderate dose of the first AED is tolerated but not wholly effective.

The outcome of localisation-related symptomatic/cryptogenic epilepsies was investigated more specifically in a separate study (Part II; Section 4). Even within this patient sub-population, response to AED treatment was determined by the nature of the underlying lesion, with MTS-related seizures being the most pharmacoresistant. This finding may lend further support to the proposition that "refractory" epilepsy is present at the outset in some patients. However, marked variability in response to treatment was observed within any given underlying pathology, so that some patients remained seizure-free after withdrawal from AED therapy whereas others continued to have seizures despite taking polypharmacy with up to four AEDs. This suggests that factors other than aetiology also affect outcome.

On the other hand, the step-wise reduction in the probability of remission with successive treatment manipulations (Figure 7), the cellular changes associated with recurrent seizures recognised in both human epilepsy and animal models, and the psychosocial consequences of chronic epilepsy (see Overall Discussion and Conclusion) suggest the possible presence of a progressive deterioration, at least for those patients suffering from recurrent seizures despite AED treatment. Hence 60% of newly diagnosed patients became seizure-free on their first (47%) or second (13%) AED, but only 1% on the third monotherapy choice (Table 11). The seizure disorder in these patients, with time, had become resistant to multiple AEDs with a range of different mechanisms of action, a situation not dissimilar to multidrug resistant

cancers (Part III; Section 1.2.1). The possibility of a progressive biochemical process associated with seizures is explored in laboratory-based studies in Part III.

6.3 A unifying hypothesis

A unifying hypothesis may be formulated from these seemingly opposing propositions. This series of observational studies suggest that patients with newly diagnosed epilepsy can be divided into two distinct populations. Around 60% will control on monotherapy. Some will remain in remission after withdrawal of AED therapy. This is supported by the consistently similar remission rate reported in outcome studies conducted in many different countries (Part I; Section 4.2) with distinctly different local preference of AED choice (e.g. PHT is widely used as the first drug in the US but much less favourable in Europe), and the similar effectiveness of the established first-line AEDs in comparative trials (Part I; Section 3.3.2). Up to 40% have "difficult-to-control" epilepsy *de novo*, as determined by the underlying aetiology or syndrome. The challenge facing the clinician is to prevent these patients from developing the full-blown state of refractory epilepsy or "multidrug resistant" epilepsy with attendant pharmacoresistance, irreversible neuronal changes, cognitive deterioration and psychosocial dysfunction. Under this unifying hypothesis, such "refractory epilepsy" is an "irreversible", but potentially preventable state by early employment of effective therapeutic interventions.

These interventions may include epilepsy surgery and rational AED combinations. In selected patient groups, the most well defined of which is MTLE associated with MTS, resective surgery can render up to 90% seizure-free (Part I; Section 3.4). There is now

accumulating evidence that AED polytherapy based on mechanisms of action may improve effectiveness when seizures are not controlled on a single drug (Deckers et al, 2000). In the observational study described in Part II; Section 3, duotherapy was well tolerated, probably attributable to the use of the newer AEDs, some of which have a more favourable side effect profile than their older counterparts (Part I; Section 3.3). Data from this study are also consistent with the view that combinations involving a sodium channel blocker and a drug with multiple mechanisms of action were more effective than others, implying that a mechanistic approach to polytherapy has potential merits.

6.4 Conclusion

Patients with symptomatic epilepsies, particularly those with MTS, and those with inadequate response to initial treatment are likely to develop refractory epilepsy. Nearly 50% of newly diagnosed patients responded to the first AED, with 90% doing so at moderate dosing. Tolerability was as important as efficacy in determining the overall effectiveness of AED treatment. Whether early surgical intervention or rational polytherapy improves long-term outcome has formed the basis of testable hypotheses that are currently being actively pursued (Engel, 1999; Part II, Section 3.5).

PART III

POTENTIAL ROLE OF MULTIDRUG RESISTANCE (*MDR*) GENE AND P-GLYCOPROTEIN IN EPILEPSY

1 General introduction

1.1 The blood-brain barrier

To exert their antiepileptic effect, AEDs need to reach and maintain an adequate concentration at their site of action, i.e. the CNS. The steady state concentration of a drug in the CNS can be regarded as an equilibrium between its rate of influx and efflux (Table 22); (Saunders et al, 1999). The mechanisms that regulate entry at the interface between the systemic circulation and the brain are collectively known as the "blood-brain barrier" (BBB).

The concept of the BBB began to develop at the end of the 19th century, pioneered by the German pharmacologist and physiologist Paul Ehrlich who demonstrated that upon intravenous injection, acidic dyes spread throughout the rat but the CNS remained unstained (Ehrlich, 1885). Subsequent experiments by Goldmann (1909), Spatz (1933) and Walter (1930) further suggested the additional existence of a blood-cerebrospinal fluid (CSF) barrier formed by the endothelial and epithelial lining of the choroid plexus, while the ependyma, which lines the brain tissue, represents the brain-CSF barrier. Although substances that do not pass the BBB may nevertheless reach the CNS by extravasation in the choroid plexus, the surface area available for exchange at the blood-CSF barrier is only 1/5000th of that at the BBB. The BBB, therefore, constitutes the most important regulatory interface between the brain and systemic circulation (De Boer and Breimer, 1994).

The BBB is crucial for the protection of the brain against potentially toxic substances in the blood and the maintenance of a constant internal environment in which optimal neuronal functions can take place. For instance, blood concentrations of the excitatory

Table 22. Main factors influencing drug accumulation in the brain (Van Bree et al, 1992; Saunders et al, 1999).

Influx	Efflux
Passive diffusion	Efflux drug pumps e.g. P-glycoprotein
Facilitated diffusion e.g. glucose	The "sink" effect
Active transport e.g. amino acids, gabapentin	Enzymatic metabolism and degradation
Transcytosis	

neurotransmitters glycine and glutamate are 1000-fold higher than in the extracellular space of the brain. At these concentrations both components would be neurotoxic, causing massive "electrical short circuits" and cell death (Schlosshauer, 1993).

Traditionally, the impermeability of the BBB is thought to be imposed by the unique morphological characteristics of the cerebral capillary endothelium (Johansson, 1990; Betz, 1992). Unlike non-neural ones, brain endothelial cells lack fenetrations and are joined by very tight junctions, blocking transport of large proteins and colloidal lanthanum. In addition, pinocytotic vesicles are almost absent in brain capillary endothelium (Van Bree et al, 1992; De Boer and Breimer, 1994). The endothelial cells are completely engulfed by a basal lamina, which is further covered by endfeet of astrocytes, pericytes and microglia (Schlosshauer, 1993). These mechanisms effectively block the passive diffusion of large, lipid-insoluble compounds. In order to obtain nutrients essential for CNS cell metabolism, specific carrier systems are present at the blood-brain interface. The glucose transporter is the best characterised example of facilitated diffusion without energy consumption (Schlosshauer, 1993). Many amino acids and some drugs, including baclofen and GBP, are actively transported by relatively specific systems (Van Bree et al, 1992). Transcytosis has been proposed as a transfer mechanism for various proteins through the BBB, including that of iron-loaded transferrin (Broadwell, 1989).

However, many molecules, including drugs, exhibit much lower BBB permeabilities than would be predicted on the basis of their small size or high lipid solubility, suggesting the presence of additional mechanism that restricts their entry into the brain. It is now realised

that drug transporter proteins, the prototype of which is P-glycoprotein (P-gp), are constitutively expressed by cerebral endothelial cells (Figure 17) where they function as active efflux pumps to transport substrate compounds back into the capillary lumen (Cordon-Cardo et al, 1989; Saunders et al, 1999). Many hydrophobic but large (>500 Da) molecules are also recognised to be substrates of P-gp, providing another explanation for their inability to penetrate the BBB (Van Asperen et al, 1997).

1.2 P-glycoprotein and the *MDR* genes

1.2.1 P-glycoprotein and multidrug resistance in cancer

Much of the present knowledge about P-gp has been gained in cancer research due to its role in chemoresistance in cancer treatment (Gottesman and Pastan, 1993). The investigation of the phenomenon of multidrug resistance, in which cancer cells are resistant to multiple anti-cancer drugs, may be traced to Burchenal's initial observations some 50 years ago (Roepe, 2000). Selection of cancer cell lines using a single cytotoxic compound leads to cross-resistance to a broad spectrum of structurally and functionally unrelated agents. The Danish physicist Danø (1973) subsequently hypothesised the presence of an active outward directed drug pump in tumour cells resistant to multiple drugs but originally exposed to only 1 or 2 anti-cancer agents. Juliano and Ling (1976) later identified a large (170 kD) membrane protein in drug resistant cells which they designated "P-glycoprotein", attributing reduced drug accumulation in its presence to impaired permeability. The P-gp genes in human and rodents have subsequently been cloned and named the *MDR* genes (Ueda et al, 1987).

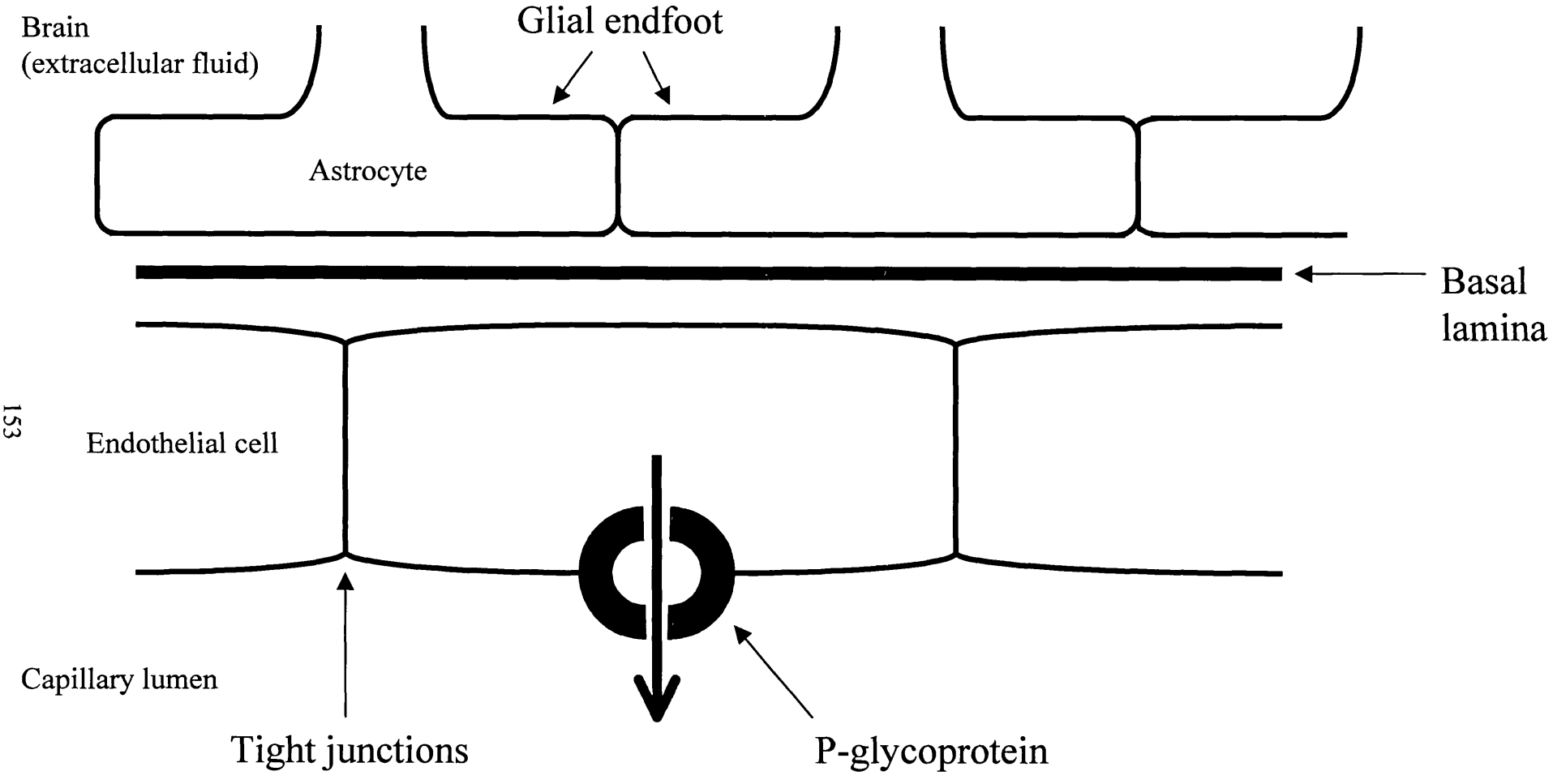


Figure 17. Factors contributing to the integrity of the blood-brain barrier.

Although other cellular mechanisms are now recognised to contribute to the multidrug resistant phenotype (Table 23), P-gp remains the most extensively investigated and best understood. Three classes of human cancers express P-gp: (a) those derived from tissues in which *MDR1* gene is constitutively activated; (b) those that have acquired multidrug resistance after repeated exposure to chemotherapeutic agents; and (c) those in which the neoplastic transforming event *per se* leads to novel activation of the *MDR1* gene (Gottesman and Pastan, 1993). The presence of P-gp in tumour cells correlates with poor chemotherapeutic response and poor prognosis, and its absence with good prognosis (Chan et al, 1991; Ling, 1997). The level of P-gp expression has been shown to be higher in subsequent relapses, and may be present in relapses if not detected at first presentation (Ling, 1997). The most persuasive evidence of P-gp's causative role in multidrug resistance comes from gene transfer experiments, in which transfection of murine or human *MDR* gene into drug-sensitive cells is sufficient to result in cross-resistance to multiple chemotherapeutics (Silverman, 1999). The level of drug resistance in *MDR*-transfected cells correlates with the density of P-gp in the plasma membrane (Choi et al, 1991). P-gp is believed to confer the multidrug resistance phenotype by reducing intracellular drug accumulation through its function as an active efflux pump (see below).

Subsequent to the discovery of P-gp, it became apparent some multidrug-resistant tumours and cell lines do not express P-gp and that other drug transporters may be involved. This observation led to the identification and cloning of the multidrug resistance protein (MRP, also called multidrug resistance-related or associated protein); (Cole et al, 1992), which has since evolved into a family of at least six members (Borst et al, 1999). Although also capable

Table 23. Cellular mechanisms contributing to multidrug resistance in cancer treatment (List, 1997).

Action	Mechanism
Extracellular drug reflux	P-glycoprotein
	Multidrug resistance protein
	P95
Intracellular entrapment or redistribution	Multidrug resistance-related protein
	Lung-resistance protein
	Transporter of antigenic peptides
Drug detoxification	Glutathione-S transferase
	Heat-shock proteins
Altered nuclear target	Topoisomerase II
	DNA repair enzymes
Altered apoptotic response	P53
	Bcl-2

of conferring multidrug resistance, *MRP1* differs from P-gp in substrate specificity (Seelig et al, 2000), structure, tissue distribution and possible physiological functions (Regina et al, 1998; Cole and Deeley, 1998; Rappa et al, 1999). Studies to detect its presence in the BBB have yielded conflicting results (Regina et al, 1998; Seetharaman et al, 1998). It may well be that there are other transporter families responsible for drug resistance awaiting discovery.

1.2.2 P-glycoprotein as a member of a transporter superfamily

P-gp and MRP are now recognised to belong to a highly conserved protein superfamily, the ATP-binding cassette (ABC) proteins which form one of the largest protein families with over 100 members found in every kind of organism examined so far, including bacteria, plants, fungi and animals (Van Veen and Konings, 1997). Thirty-nine human ABC proteins have been identified, half of which were discovered through progress of the Human Genome Project in the past several years (Klein et al, 1999). With few exceptions, the human ABC proteins function as active pumps at the cell membrane through hydrolysis of ATP to drive the flux of their substrate against a concentration gradient. The substrate range for these proteins is diverse and includes drugs, nutrients, amino acids, sugars, peptides, pigments, and metals (Silverman, 1999). These transporters share a common organisation and considerable amino acid homology around the nucleotide-binding domain (see below), suggesting evolutionary conservation. Examples of other ABC proteins include *pfmr*, which mediates resistance in the malarial parasite *Plasmodium falciparum*, and the antigenic peptide transporter involved in MHC antigen presentation (Higgins, 1992; Lepage and Gros, 1993). Mutations in these proteins are causative in a range of genetic disorders, such as the cystic

fibrosis transmembrane conductance regulator (CFTR) in cystic fibrosis, MRP2 in Dubin-Johnson syndrome, and adrenoleukodystrophy protein (ALDP) in ALD (Klein et al, 1999).

1.2.3 Genetics, structure and mechanism of action of P-glycoprotein

P-gp is encoded by a small gene family, comprised of two genes in human, designated *MDR1* and *MDR2*, located near each other on chromosome 7q21.1 (Callen et al, 1987), and three genes in rodents, *mdr1a, mdr1b* and *mdr2* (Silverman, 1999; Table 24). Based on sequence identity and function, mammalian *MDR* genes are classified into two categories (Borst, 1997; Silverman, 1999). Class I genes (human *MDR1,* rodent *mdr1a* and *1b*) encode the drug transporter associated with multidrug resistance, whereas the class II genes (*MDR2, mdr2*) do not confer the multidrug resistance phenotype but encode a phosphotidylcholine transporter in biliary canaliculi. The *MDR* genes are highly conserved. Sequence comparison shows considerable identity and homology amongst them (Brown et al, 1993). For example, the human *MDR1* and *MDR2* coding sequences are 75% identical despite different transport properties, and the human *MDR1* and mouse *mdr1a* genes, with similar function, are 82% identical, despite 50 million years or more of evolution. For the purpose of this thesis, unless otherwise specified, the term P-gp refers to that encoded by the class I *MDR* genes, which is used to include human *MDR1* and both *mdr1a* and *mdr1b* in rodents.

The molecular weight of P-gp varies slightly from species to species, and from gene to gene. Human P-gp encoded by the *MDR1* gene is an integral membrane protein with 1280 amino acids and a molecular weight of approximately 170 kDa (Dicato et al, 1997). Similar to other ABC transporters, sequence analysis suggests it comprises two homologous halves, each

Table 24a. The mammalian multidrug-resistance gene family. (Alternative nomenclature in parentheses.)

Species	Class I	Class II
Human	*MDR1*	*MDR2 (MDR3)*
Rat	*mdr1a, mdr1b*	*mdr2*
Mouse	*mdr1a (mdr3), mdr1b (mdr1)*	*mdr2*
Hamster	*pgp1, pgp2*	*pgp3*

Table 24b. GenBank accession numbers of *MDR* cDNA sequences *.

Gene		Accession number	References
Human	*MDR1*	M14758	Chen et al, 1986
	MDR2	M23234	Van der Bliek et al, 1988
Rat	*mdr1a*	AF257746	Unpublished
	mdr1b	M62425	Silverman et al, 1991
	Mdr2	L15079	Brown et al, 1993
Mouse	*mdr1a*	M30697, M33581	Devault and Gros, 1990
	mdr1b	M14757	Gros et al, 1986
	Mdr2	J03398	Gros et al, 1988
Hamster	*mdr1a*	M60040	Endicott et al, 1991
	mdr1b	M60041	Endicott et al, 1991
	Mdr2	M60042	Endicott et al, 1991

* Complete intron-exon structure known for human *MDR1* (Chen et al, 1990) and *MDR2* (Lincke et al, 1991) only.

consisting of one transmembrane domain containing six segments, and one nucleotide-binding domain or ABC unit (Figure 18; Gottesman and Pastan, 1993; Higgins et al, 1997; Klein et al, 1999). Recent analysis by high resolution electron microscopy suggests a three-dimensional structure as a monomer with a 5-nm central pore closed on the cytoplasmic surface of the plasma membrane forming an aqueous compartment (Rosenberg et al, 1997). The energy of ATP hydrolysis is utilised by a mechanism that involves positive cooperativity between the two ABC units and a tight molecular coupling of the transmembrane domains to the ABC units, so that conformational changes caused by substrate binding is transmitted to active transport (Klein et al, 1999).

The mechanism of action of P-gp remains hotly debated (Roepe, 2000). The initial hypothesis of altered plasma membrane permeability (Juliano and Ling, 1977) soon gave way to direct efflux pump activity (Willingham et al, 1986) whereby substrates are actively extruded from intracellular to extracellular space against the concentration gradient (Sharom, 1997). Although such a classical pump model explains most of P-gp's mechanisms of action, it cannot account for the protein's unusually broad substrate specificity in contrast to other membrane transporters (Roepe, 2000). This led to the suggestion that hydrophobic drugs are removed directly from within the lipid bilayer of the plasma membrane rather than from the aqueous phase (Gottesman and Pastan, 1993; Shapiro and Ling, 1998). This "hydrophobic vacuum cleaner" model may account for P-gp's wide range of substrates which are all hydrophobic and amphipathic. A further modified version of the pump model (the "flippase" model) which is gaining increasing support suggests that P-gp may interact with substrates in the inner leaflet of the lipid bilayer and "flip" them to the outer leaflet (Ueda et al, 1997; Borst

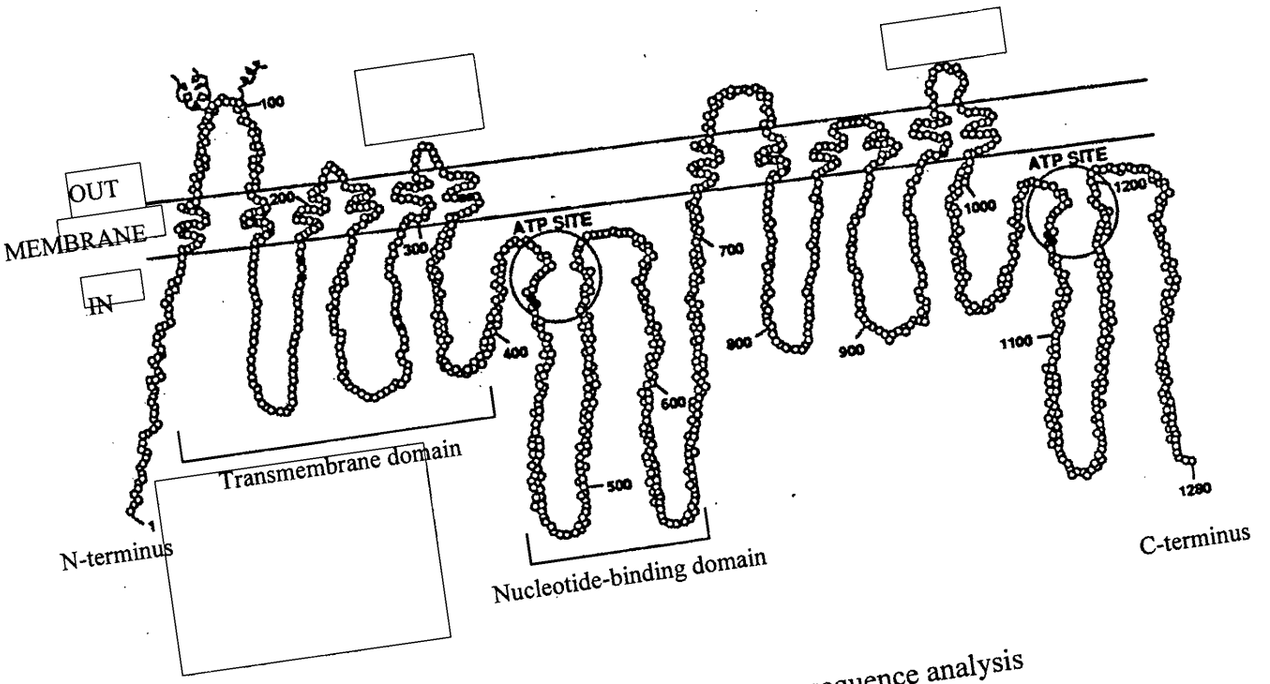


Figure 18. Model of human P-glycoprotein derived from sequence analysis (modified from Chen et al, 1986)

and Schinkel, 1997). Suggestion of indirect secondary effects of P-gp on passive intracellular drug accumulation by altering intracellular pH or membrane potential (Roepe, 2000) has not been widely accepted (Borst and Schinkel, 1997). It remains unclear how many drug-binding sites or pores P-gp possesses.

1.2.4 Substrates and modulators of *MDR1* P-glycoprotein

Unlike most energy-dependent pumps, P-gp appears to be highly promiscuous. Hundreds of structurally and chemically unrelated compounds, as diverse as anthracyclines (doxorubicin), alkaloids (vincristine), specific peptides (cyclosporin A), steroid hormones (hydrocortisone), local anaesthetics (dibucaine), dye molecules (rhodamine 123), to mention only a few, have been identified as substrates (Table 25). How P-gp recognises such a diverse range of compounds is still largely unexplained (Ueda et al, 1997). It has been said that a "typical" substrate is large (molecular weight > 400), hydrophobic, amphipathic, with a planar ring system and a weak positive charge at physiological pH (Sharom, 1997). However, transported compounds can range in size from 250 Da (cimetidine) to almost 1900 Da (gramicidin D), and apart from aromatic groups, non-aromatic linear or circular compounds can also be substrates, and some alkaline drugs (e.g. methotrexate) are transported (Schinkel, 1997). Amphipathy and hydrophobicity which allow intercalation into the membrane lipid bilayer appear to be the only common characteristics among the substrates (Gottesman and Pastan, 1993; Schinkel, 1997; Ueda et al, 1997). Although it has been suggested recently that the number of electron donor groups and spatial separation may be useful in predicting likely P-gp substrates (Seelig, 1998), the possibility of a compound being a substrate cannot be ruled out until adequately tested.

Table 25. Examples of P-glycoprotein substrates and inhibitors (Ford et al, 1996; Sharom, 1997; Seelig, 1998; Rodriguez et al, 1999).

P-gp substrates			
Anticancer drugs	**Other cytotoxic agents**	**Steroids**	**Miscellaneous**
Doxorubicin	Colchicine	Aldosterone	Digoxin
Daunorubicin	Emetine	Dexamethasone	Protease inhibitors
Vinblastine	Actinomycin D		Loperamide
Vincristine	Puromycin	**Peptides**	Rhodamine 123
Etoposide	Mitoxantrone	Leupeptin	^{99m}Tc-SESTAMIBI
Teniposide		Pepstatin A	Triton X-100
Paclitaxel		Gramicidin D	
Docetaxel		Nonactin	

P-gp inhibitors			
Calcium channel blockers	**Cyclic peptides**	**Steroids**	**Miscellaneous**
Verapamil	Cyclosporin A	Progesterone	Dipyridamole
Nifedipine	Valinomycin	Tamoxifen	Amiodarone
Diltiazem	Tacrolimus	Cortisol	Quinidine
Nicardipine			Propranolol
	Phenothiazines	**Antibiotics**	Chloroquine
	Trifluoperazine	Erythromycin	Terfenadine
	Chlorpromazine	Ceftriaxone	Reserpine

Ever since the role of P-gp was recognised in drug resistance in cancer, modulators (also called chemosensitisers, inhibitors or reversal agents) to circumvent its functions as a means to overcome resistance have been sought (Ford and Hait, 1993). This has generated another bewildering list of compounds which increase the sensitivity of cells *in vitro* to cytotoxic substrate drugs (Table 25). Many of these modulators are themselves substrates for P-gp, suggesting they act by competitive inhibition (Ford et al, 1996). "First-generation" modulators include drugs already developed for other therapeutic indications, such as calcium channel blockers (verapamil), immunosuppresants (cyclosporin A), antiarrhythmic drugs (quinidine, amiodarone), and steroidal agents (progesterone, tamoxifen), some of which have been evaluated in phase II/III clinical trials as adjuctive agents in solid and haematological cancers (Ford and Hait, 1993; Ford et al, 1996; Silverman, 1999). Their results have so far been disappointing, partly because of dose-limiting side effects due to their low affinity (Dicato et al, 1997). In other instances, toxicity of the chemotherapeutic agent is increased due to pharmacokinetic interactions with the P-gp modulator (Dalton, 1994; Lum and Gosland, 1995), either through metabolism or absorption and disposition which are now recognised to be significantly influenced by P-gp. In addition, other drug transporters and cellular mechanisms may be responsible for the multidrug resistant phenomenon (Table 23), thus a P-gp reversing approach alone may not be sufficient to improve treatment outcome (Dicato et al, 1997). Less toxic, "purpose-made" second-generation inhibitors (e.g. PSC 833) which are effective at much lower concentrations as well as other more sophisticated ways to modulate P-gp functions are being developed (Sarkadi and Müller, 1997).

1.2.5 Tissue distribution and physiological functions of P-glycoprotein

The tissue distribution of a protein can yield important clues to its function. Measurements of *MDR1* RNA (Fojo et al, 1987) and P-gp protein levels (Sugawara et al, 1988; Cordon-Cardo et al, 1990) have reported high degree of expression in organs with excretory functions, such as the adrenal cortex, epithelial cells on the lumenal surface of biliary hepatocytes, pancreatic ductules, small and large intestine mucosal cells and proximal renal tubules (Table 26). Another group of anatomical sites with high level of *MDR1* expression is the blood-tissue barriers, in particular the capillary endothelial cells of the brain (Cordon-Cardo et al, 1989; Beaulieu et al, 1997), testis and placenta (Sugawara et al, 1988). More recently, functional *MDR1* P-gp has also been detected in the blood-CSF barrier across the choroid plexus epithelium (Rao et al, 1999) and in the blood-inner ear barrier in mice (Zhang et al, 2000b). In addition, *MDR1* is significantly expressed by cells in the haematopoietic system, particularly the progenitor cells and CD8+ cytotoxic T cells (Schinkel, 1997).

Investigation of P-gp in the human foetus using RNase protection assays suggested expression as early as 7 weeks (van Kalken et al, 1992). Immunohistochemical analysis showed staining in kidney by 11 weeks of gestation, liver, adrenal, heart, and smooth muscle by 13 weeks, and brain, stomach, intestine, and bile ducts by 28 weeks.

In mice (and probably also in rats), the two drug-transporting *mdr1a* and *mdr1b* P-gps have overlapping but distinct substrate specificities (Devault and Gros, 1990) and tissue distributions (Croop et al, 1989), but together they cover the same tissues as the single *MDR1*

Table 26. *MDR1* P-glycoprotein in normal human tissues (Van der Heyden et al, 1995).

High expression level		**Moderate expression level**		**Low expression level**
Tissue	**Cellular localisation**	**Tissue**	**Cellular localisation**	**Tissue**
Brain	Capillary endothelium	Trachea	Apical epithelial cells	Skin
Adrenal cortex		Adrenal medulla		Skeletal muscle
Kidney	Proximal tubules	Major bronchi		Heart
Liver	Bile canalicular face of hepatocytes	Prostate	Glandular	Spleen
Placenta	Trophoblasts			Oesophagus
Colon	Luminal mucosal surface			Stomach
Small bowel	Apical brush border of luminal cells			Ovary
Pancreas	Luminal surface of small ductile epithelial cells			Spinal cord
Testis	Endothelial cells			Bone marrow

in man (Table 27), suggesting that they fulfil the same physiological role(s) in the different species (Schinkel, 1997).

In view of its vectorial transport capacity and the pattern of tissue distribution, P-gp is widely believed to play an important role in the protection of the organism against xenobiotics, either by exclusion of the toxins, as in the intestine and blood-tissue barriers, or by active excretion in the liver, intestine and kidney (Schinkel, 1997). Its expression in the adrenal gland and reproductive organs, and the observation that several corticosteroid hormones are transported by P-gp while progesterone is an inhibitor, suggest that P-gp may be involved in steroid secretion (Chin and Liu, 1994; Schinkel, 1997). The more primitive the haematopoietic cells, the higher the level of P-gp function, implying a possible role in the maturation of bone marrow stem cells (Schinkel, 1997). The early foetal expression and wide tissue distribution also suggest an important role in transport of endogenous substrates and protection from xenobiotics during a critical phase of development.

From a pharmacological point of view, judging from its strategic distribution in organs of drug absorption, metabolism and elimination, P-gp can be expected to play a potentially pivotal role in the pharmacokinetics and disposition of its substrates (Fromm, 2000). Indeed, studies using *mdr1* gene knockout mice show that active excretion by intestinal P-gp limits the oral absorption of certain substrates such as HIV-proteases (Kim et al, 1998) and paclitaxel (Sparreboom et al, 1997). P-gp and the hepatic metabolising P-450 isoenzymes, particularly CYP3A, share many common substrates and are simultaneously induced by many drugs, suggesting possible coordinate regulatory elements and that the two mechanisms may play complementary roles in drug resistance (Burt and Thorgeisson,

Table 27. Relative expression level of *mdr1a* and *mdr1b* in mouse tissues determined by RNA-DNA hybridisation (Croop et al, 1989).

Tissue	*mdr1a*	*Mdr1b*
Pregnant uterus		+++++
Adrenal		++++
Placenta		+++
Uterus		+
Kidney	+	++
Liver	+	+
Muscle	+	+
Spleen	+	+
Heart	++	++
Brain	++	+?
Lung	++	+
Intestine	+++	
Stomach	+	
Spinal cord	++	
Testes	++	

1988; Wacher et al, 1995). Impaired P-gp function was associated with an exaggerated induction of CYP3A by rifampicin (Schuetz et al, 1996). P-gp in the intestine, renal tubules and the biliary tract is responsible for the elimination of many of its substrates (Silverman, 1999). For example, delayed elimination of digoxin from the blood stream in *mdr1* knockout mice is mainly due to reduced faecal excretion (Schinkel, 1998). Finally, using *mdr1* knockout mice, it has been shown that P-gp limits the penetration of its substrates across many blood-tissue barriers, including the blood-brain barrier (Schinkel et al, 1994; Schinkel et al, 1996; Schinkel et al, 1997; Kim et al, 1998), blood-CSF barrier (Rao et al, 1999), and the blood-inner ear barrier (Zhang et al, 2000b). The contribution of *mdr1* knockout mice to the understanding of P-gp functions will be further discussed in Part III; Section 2.1.

The influence of P-gp in all key kinetic stages means that any attempt to modulate its function in one step is likely to have knock-on effects on the others. Thus, administration of inhibitors aiming to enhance tissue penetration of a P-gp substrate may also lead to raised plasma concentration due to increased oral bioavailability, reduced hepatic metabolism, and reduced elimination, causing increased systemic toxicity (Lum and Gosland, 1995).

P-gp has also been demonstrated to enhance cell volume-activated chloride currents *in vitro* (Valverde et al, 1992; Mintenig et al, 1993; Higgins, 1995). Whether P-gp possesses an additional function as a chloride channel (unlikely), or it activates an endogenous and as yet unidentified chloride channel, is unclear (Borst and Schinkel, 1997).

1.2.6 *MDR1* expression at the blood-brain barrier

It is increasingly recognised that P-gp plays a vital role in maintaining the integrity of the BBB. P-gp substrates may reach almost 100-fold higher in the brains of *mdr1a* knockout mice that are devoid of P-gp at cerebral capillary endothelium compared with their wild-type counterparts, sometimes with lethal consequence (Schinkel, 1997). Although some authors have suggested that P-gp is located at the astrocyte foot processes at the BBB (Pardridge et al, 1997), most studies report its expression at the luminal side of capillary endothelium, consistent with its presumed barrier function (Beaulieu et al, 1997; Seetharaman et al, 1998). P-gp is normally not expressed by brain parenchyma in man, but it has been detected in diseased astrocytes, e.g. in astrocytomas (Matsumoto et al, 1991), and in patients with phenylketonuria, tuberculous leptomeningitis, and encephalitis (Bietzmann et al, 1994). In addition, P-gp expression has been reported to disappear acutely following focal cerebral ischaemia in rats, but to reappear after several days (Samoto et al, 1994), indicating regulation in response to external factors.

Whether both drug-transporting *mdr1a* and *mdr1b* are expressed in rodent brains has been investigated in few studies, often involving the whole brain and employing different techniques. In rats, it is generally agreed that only *mdr1a* is present in cerebral microvessels *in vivo* (Barrand et al, 1995; Regina et al, 1998), but *mdr1b* has also been detected in brain parenchyma using reverse transcriptase-polymerase chain reaction which is more sensitive than immunohistochemistry (Regina et al, 1998). In mice, one study using DNA-RNA hybridisation found both *mdr1a* and *mdr1b* in homogenised brain tissues, although the latter at lower level (Croop et al, 1998). In addition, brain levels of P-gp substrates (e.g. digoxin) can reach one to two orders of magnitude higher in *mdr1a* knockout or *mdr1a/1b* "double-knockout" mice compared to wild-type mice, whereas

similar brain levels are found in *mdr1b* knockout mice and normal mice (Schinkel et al, 1997). This also suggests that *mdr1a* is the predominant functioning P-gp in mouse BBB. However, expression of *mdr1b* confined to certain discrete "at risk" region(s) in the rodent brain conferring "extra protection" cannot be ruled out.

1.2.7 Control of *MDR1* gene expression

Although a consistent pattern of distribution has emerged from tissue studies, the exact level of P-gp expression was highly variable between different individuals in these studies, suggesting genetic or environmental influences (Van der Heyden et al, 1995; Silverman, 1999). Recently, single nucleotide polymorphisms of the *MDR1* gene have been described, some of which were preferentially expressed by multidrug resistant cell lines (Mickley et al, 1998). Sequence analyses covering the entire gene in 24 healthy volunteers identified a relatively common polymorphism in *MDR1* (homozygous in 24% of test subjects) which was associated with changes in P-gp protein level and activity *in vivo*. In addition, another polymorphism was present just preceding the translation start codon and might influence initiation, while three were associated with amino acid changes with possible functional consequences (Hoffmeyer et al, 2000). Identification of nucleotide polymorphisms has raised the possibility of pharmacogenetic influence in individual response to treatment with P-gp substrates both in terms of efficacy and tolerability (see Part I; Section 5.2.2).

Expression of P-gp is highly inducible by environmental factors. Novel activation of the *MDR1* gene can be found in neoplasms derived from tissues not normally expressing the gene, or in tumours during relapse but not on initial presentation (Gottesman and Pastan, 1993). Apart from its substrate drugs, *MDR1* expression has been found to be induced by heat shock, arsenite, partial hepatectomy, extracellular matrix, growth factors, sodium

butyrate, protein kinase C agonists, and even its inhibitors such as verapamil (Gottesman et al, 1995). The process of cell culture has been shown to lead to an increase in the expression of *mdr1b* in rat brain endothelial cells, with reduction in that of *mdr1a* (Barrand et al, 1995).

Although drug resistant cell lines selected *in vitro* usually display an amplification of DNA, elevation of mRNA and P-gp is often observed in both human and animal tumours without concomitant amplification of the *MDR1* gene (van der Heyden et al, 1995). The primary mode of regulation of expression in clinical conditions *in vivo* thus appears to be at the transcriptional level, either by increasing the amount of new RNA transcripts or by enhancing their stability (Thorgeirsson et al, 1994). Post-translational modifications by glycosylation and phosphorylation are not required for the basal transport activity of P-gp but may affect its functional properties such as substrate affinity and efflux velocity (Gottesman et al, 1995). In addition, the possibility of co-amplification of linked or non-linked genes capable of altering drug response (e.g. MRP) needs to be considered. These factors may explain the variation in the pattern of drug resistance seen in different selection schemes of multidrug resistant cell lines, even under conditions in which the overexpression of *MDR* genes gives a wild-type phenotype.

1.2.8 Measurement of *MDR1* gene expression

Since gene amplification is thought not to be relevant in the development of clinical drug resistance, the determination of *MDR1* expression has centred primarily on the detection of messenger ribonucleic acid (mRNA) or P-gp, for which a number of methods have been developed (Van der Heyden et al, 1995). Techniques to detect mRNA include Northern blot, slot-blot analysis, in situ hybridisation, RNase protection assay, and reverse

transcriptase-polymerase chain reaction (RT-PCR). Compared with the other techniques, RT-PCR is highly sensitive and requires much less tissue. In addition, by using an internal standard to compete with the target, the absolute amount of mRNA molecules can be measured, whereas only a relative comparison can be made with other methods. RT-PCR is, therefore, preferable when quantification is required but only a small sample is available (Zhang et al, 1996a; Zhang et al, 1996b). The theoretical basis of quantitative RT-PCR will be further discussed in Part III; Section 3.1.

One of the major drawbacks of all RNA detection techniques is their labour intensive nature. In addition, they do not allow localisation of gene expression or distinction between normal and abnormal tissues. In contrast, immunohistochemistry permits detection of P-gp at single cell level, which is not possible by Western blot (Friedlander et al, 1989). However, protein detection techniques are generally time-consuming and require large samples. The staining antibody may cross-react with other molecules and a panel of antibodies is needed to improve reliability and specificity (Van der Heyden et al, 1995). Sample preparation may also affect the detection rate of P-gp (Cordon-Cardo et al, 1990), and only semiquantitative measurement is possible by Western blot or immunohistochemical staining.

A third alternative to measure P-gp expression is functional assays, the most commonly employed of which is flow cytometry using monoclonal antibodies or specific dyes such as rhodamine-123 in selected cell lines (Davies et al, 1996). This technique can be applied in routine clinical screening as it is widely available, cheap, reproducible, and easy to perform. However, flow cytometry requires reliable cell suspensions, which often results in substantial cell damage. Another problem is the low specificity of the labelling techniques

and factors other than drug efflux may contribute to the measured function (Van der Heyden et al, 1995). Recently, visualisation of P-gp using SPECT or PET has been developed, most commonly using [^{99m}Tc]SESTAMIBI or other radiolabelled substrates as ligands (Hendrikse et al, 1999). These techniques may offer a non-invasive way to monitor the dynamic function of the P-gp drug efflux pump *in vivo*.

1.3 P-glycoprotein and "multidrug resistant epilepsy" - the hypothesis

As eluded above, although the investigation of P-gp and its other drug transport members originated from research in oncology, it is increasingly recognised to significantly influence the safety and efficacy of many other non-cancer agents. It is conceivable that P-gp may also play a role in pharmacoresistance in epilepsy, analogous to drug resistance in cancer. Up to one third of patients with epilepsy continue to have seizures despite AED treatment, and the cause of this "refractoriness" is largely unknown (Part I; Section 5.2). In these patients, the seizure disorder displays resistance to multiple agents, either singly or in combination, with a variety of modes of action, suggesting the operation of a universal mechanism affecting AEDs in a general fashion. One possible candidate mechanism might be limitation of access of AEDs to the brain due to the efflux action of P-gp at the BBB. AEDs, which require penetration across the BBB to exert their antiepileptic effect, might be prevented from accumulating to a sufficiently high concentration, either in the brain in general or specifically in the seizure focus, if they were extruded by P-gp located at the cerebral capillary endothelium. If the function of the BBB was enhanced around the epileptogenic focus by increased P-gp expression, AEDs might be prevented from entering their site of action, but could still penetrate the rest of the brain to cause neurotoxicity. This might also explain why symptomatic epilepsies (with structural lesions) are more drug-resistant than idiopathic epilepsies (usually genetic with presumably more diffuse

changes), and why certain lesions (e.g. MTS) are particularly so if such changes were more prominent in specific brain regions. Furthermore, if P-gp were induced by neuronal discharges, in much the same way as heat shock or other external factors, a vicious cycle might ensue whereby more P-gp leads to further uncontrolled seizures, ultimately resulting in "multidrug resistant epilepsy". If so, P-gp inhibitors might potentially be given together with AEDs to increase their access to the desired site of action and improve efficacy, offering a truly "rational" approach to combination therapy.

Such a proposition is based on two separate but parallel hypotheses. First, and perhaps most importantly, some of the available AEDs must be substrates of P-gp. If not so, any elevation in P-gp expression would be unlikely to have clinical relevance. *In vitro* analysis (Tishler et al, 1995; Seling, 1998; Schinkel et al, 1996) suggest PHT is transported by P-gp. Other established and new AEDs have not been tested, but their hydrophobicity makes them candidate-P-gp substrates. Although they do not all conform to the "typical" planar ring structure, such possibility cannot be excluded until they are adequately tested (Part III; Section 1.2.4). If they were indeed P-gp substrates, the possibility that they are also inducers of P-gp would merit exploration.

Second, increased expression of *MDR1* is associated with refractory epilepsy. Evidence to support this has come from examination of brain tissues resected from patients with refractory epilepsy. Tishler and colleagues (1995) studied *MDR1* expression in 19 patients undergoing epilepsy surgery. The median time from seizure onset to surgery was 19 years (range 3-41 years). Treatment with PHT had been unsuccessful in all, CBZ in 18, PB in 15, and VPA in 12. Temporal lobe resections were performed in 15 of the 19 patients, sclerosis was observed in 13 specimens, tumours in 4, carvenous angioma in 1 and

inflammation in the remaining patient. Using a semiquantitative RT-PCR technique, *MDR1* mRNA level was found to be over 10 times higher in 11 of the 19 resected samples compared with controls ("normal" brain tissues resected during removal of arteriovenous malformations). In addition, P-gp was detected by immunohistochemical staining in astrocytes where it is not normally present, suggesting novel expression similar to other conditions with diseased astrocytes (Part III; Section 1.2.6) and malignant tumours (Part III; Section 1.2.1). The small number of study subjects did not allow exploration of mechanisms controlling expression, such as seizures or underlying lesions or previous AED treatment.

Since this report, sporadic case series investigating the association between P-gp and epilepsy have trickled through the literature. Published as an abstract, D'Giano and colleagues (1997) examined P-gp by immunohistochemistry in patients undergoing epilepsy surgery for refractory TLE. P-gp was stained in neuronal and glial components in 9 out of 12 resected temporal lobe specimens in the region of MTS but not in the lateral neocortex. Quantification or comparison with control tissues was not undertaken. The same group of investigators later reported a single case of detection of P-gp (again by antibody staining) in tuber cells removed from a 4-month-old infant with tuberous sclerosis and uncontrolled epilepsy (Lazarowski et al, 1999). Sisodiya and co-workers (1999) used two monoclonal antibodies to stain P-gp in post-mortem archival samples of a range of cortical dysplasias (CD). Positive perivascular glial labelling was noted in 10 of 16 CD samples but only 2 of 16 age-matched controls. Although none of the CD patients (aged 20 weeks to 7 years at post-mortem) had suffered seizures, they were regarded as "pre-epileptic" by the authors since most patients with CD are thought to develop epilepsy eventually.

1.4 Summary

Over-expression of the drug transporter P-gp, encoded by the *MDR1* gene, confers chemoresistance in certain cancers. P-gp extrudes a wide range of xenobiotics from cells and is present in cerebral capillaries where it contributes to the integrity of the BBB. Over-expression of *MDR1* has been reported in brain tissues removed from patients with refractory epilepsy. It is hypothesised that some AEDs are substrates of P-gp and increased P-gp expression is associated with drug resistance in epilepsy.

A series of laboratory studies were carried out to test these hypotheses. *Mdr1a* knockout mice were used to determine whether any of the established and new AEDs are subject to transport by P-gp. To determine gene expression, cerebral *mdr1* mRNA concentrations in animal models of seizure and tissue damage as well as human specimens were measured with use of specifically constructed competitive internal standards. The specific aims of these studies and the rationale behind the various models will be discussed in detail in the individual sections.

2 Pharmacokinetics of antiepileptic drugs in *mdr1a* knockout mice

2.1 Introduction

Mdr1a knockout (*mdr1a* (-/-)) mice of FVB background were generated by Schinkel and colleagues using embryonic stem cell technology (Schinkel et al, 1994). Subsequently, *mdr1b* (-/-) mice and mice deficient in both *mdr1a* and *mdr1b* genes (*mdr1a/1b* (-/-) mice) have been produced and studied (Schinkel et al, 1997). Each of these strains has so far shown no difference in viability, health, weight, fertility and litter size from their wild-type (+/+) counterparts. Extensive analyses of serum biochemical and haematological parameters, and macroscopic and microscopic surveys of all major organs have revealed no abnormalities (Schinkel, 1997; Schinkel, 1998).

However, these mice exhibit major abnormalities in drug handling. The *mdr1a* (-/-) mice have been most extensively studied. Drugs that are substrates of P-pg reach significantly higher levels in organs that normally express *mdr1a*, particularly in the brain. The most dramatic example is with ivermectin, a generally safe pesticide routinely used to treat mite infestation. At 24 hours after oral administration, its level in the brain was nearly 90 fold higher in *mdr1a* (-/-) mice than in *mdr1a* (+/+) mice with lethal consequence. A toxicity assay demonstrated that *mdr1a* (-/-) mice were 50 to 100-fold more sensitive to the toxic effect of ivermectin than wild-type mice (Schinkel et al, 1994). Similar increased accumulation in the brains of *mdr1a* (-/-) mice has been observed for a variety of drugs such as digoxin, vinblastine (Schinkel et al, 1994; Schinkel, 1998), loperamide, ondansetron (Schinkel et al, 1996), HIV-1 protease inhibitors (Kim et al, 1998), quinidine (Kusuhara et al, 1997), and amitriptyline (Uhr et al, 2000).

The *mdr1a* (-/-) mice are, therefore, gaining popularity as a useful tool for the screening of P-gp substrates, particularly at the BBB level. There are, however, several caveats to their use. Firstly, in addition to effects on drug passage across blood-tissue barriers, complex pharmacokinetic consequences can be anticipated in view of the wide tissue distribution of P-gp (Part III; Section 1.2.5). Higher plasma levels of P-gp substrates (e.g. digoxin, paclitaxel) have been observed in *mdr1a* (-/-) mice, an effect attributed to increased intestinal absorption or reduced elimination. Changes in cytochrome P450, in particular CYP3A, have also been reported in *mdr1* knockout mice (Schuetz et al, 2000), potentially affecting drug metabolism. Thus any increase in brain concentration needs to be interpreted in the light of changes in plasma and other organs.

The role of *mdr1b* has not been fully explored. The tissue levels of digoxin in *mdr1b* (-/-) were similar to those in the wild-type mice, whereas *mdr1a/1b* (-/-) mice had higher levels comparable to *mdr1a (-/-)* mice (Schinkel et al, 1997). Although such observation may be interpreted as consistent with the general view that *mdr1a* is the only or predominant *mdr1* gene expressed in the mouse brain, P-gps encoded by the two isoforms do have distinct drug specificities (Devault and Gros, 1990), and localised expression of *mdr1b* in the brain cannot be ruled out (see Section 1.2.6). Substances that are not transported by P-gp in *mdr1a* (-/-) mice should, therefore, be screened in *mdr1b* (-/-) mice before concluding that they are not substrates of murine P-gp. Such concern does not arise in man in whom there is only one drug-transporting P-gp encoded by *MDR1*.

The *mdr1* knockout mice appear to be indistinguishable from their wild-type counterparts as long as they are not challenged with drugs. This might suggest that drug-transporting P-gps have no essential physiological functions. However, the absence of P-gps *ab initio*

might allow the development of compensatory mechanisms that obfuscate the detection of essential P-gp functions. For instance, the expression of *mdr1b* is upregulated in the liver and kidney (but not in other tissues) of *mdr1a* (-/-) mice (Schinkel et al, 1994). So far examination of other transporters such as *mrp, Sister of P-gp, oct1* and *cftr* has not revealed evidence of compensatory upregulation in *mdr1a/1b* (-/-) mice (Schinkel et al, 1997), although these represent only a very small fraction of possible candidate transporters.

2.2 Aims

The aim of this pilot study was to examine whether the established and new AEDs are substrates of mouse *mdr1a* P-gp by comparing their pharmacokinetics in *mdr1a* (-/-) mice and *mdr1a* (+/+) mice. Based on previous observations of large difference in brain levels for other P-gp substrates, *mdr1a* (-/-) mice would be particularly suited to examine whether P-gp at the BBB limits CNS penetration of AEDs which obviously require brain access to exert their desired pharmacological effects. AEDs that are substrates of P-gp can be expected to reach higher brain levels in *mdr1a* (-/-) mice than in wild-type mice.

2.3 Methods

2.3.1 Materials

Female *mdr1a* (-/-) mice and *mdr1a* (+/+) FVB mice between 8 and 20 weeks of age (20-30g) were used. They were bred in-house by Dr Elizabeth CM de Lange at Leiden/Amsterdam Center for Drug Research and kept in a controlled temperature and humidity environment with day/night cycle conditions and access to food and water *ad libitum*.

The established AEDs, PB, PHT, CBZ and VPA, were purchased from Sigma Chemical Company (Poole, UK). The new AEDs were obtained from the following companies: LTG from GlaxoWellcome Research and Development (Stevenage, UK), TPM from RW Johnson Pharmaceutical Research Institute (Spring House, PA, USA), GBP from Parke-Davis Pharmaceutical Research (Ann Arbor, MI, USA), and VGB from Hoechst Marion Roussel (Uxbridge, UK).

2.3.2 Drug administration

All drugs were prepared on the day of use. CBZ was dissolved in 30% (v/v) glycofurol (Taubøll et al, 1990), while the other seven drugs were all dissolved in 0.9% saline. The pH was adjusted to 9.0 with NaOH for PHT and TPM to improve solubility. Seventy-two *mdr1a* (-/-) and 72 *mdr1a* (+/+) mice were randomised into eight treatment groups (n = 9/group/genetic type) and AEDs were administered subcutaneously. The following doses of drugs were injected: PB, 20mg/kg; PHT, 20mg/kg; CBZ, 20mg/kg; VPA, 200mg/kg; LTG, 5mg/kg; TPM, 50mg/kg; GBP, 50mg/kg; VGB, 500mg/kg. Drugs were formulated to ensure uniformity of injection volume.

Three mice from each treatment group of each genetic type were sacrificed by decapitation at 30 minutes, three at 60 minutes, and three at 240 minutes after drug administration. A truncal blood sample was obtained and the brain, liver, heart and skeletal muscle (thigh) removed. Tissues were rinsed with 0.9% saline and dried by blotting to remove excess blood. The samples were stored at –70°C until required.

2.3.3 Sample preparation

Blood samples were centrifuged at 800x*g* for 10 minutes to obtain serum. Tissue samples for CBZ, PHT, VPA and PB analysis were homogenised in 5 volumes (w/v) of 0.9% saline and centrifuged (800x*g*) for 5 minutes. Supernatants were decanted into glass tubes and 5ml diethylether / dichlorormethane (3:1) was added. Tubes were vortex-mixed and centrifuged for a further 5 minutes (800x*g*). The organic layer was removed and evaporated to dryness. Residues were reconstituted in 120µl of synthetic plasma for analysis. TPM tissue samples were homogenised in 5 volumes (w/v) of EDTA buffer (Sills et al, 2000) and centrifuged (800x*g*) for 15 minutes. The supernatant was decanted, re-spun (800x*g*) for a further 15 minutes and the resulting supernatant analysed. GBP tissue samples were homogenised in 5 volumes (w/v) of 1% perchloric acid and LTG and VGB samples homogenised in 5 volumes (w/v) of 0.9% saline. All homogenates (GBP, LTG, VGB) were then centrifuged (800x*g*) for 15 minutes prior to analysis.

2.3.4 Drug assays

CBZ, PHT, VPA and PB samples (serum and extracted tissue) were analysed by enzyme mediated immunoassay technique (EMIT, Syva Company, Cupertino, CA, USA). Serum and tissue TPM concentrations were determined by fluorescence polarisation immunoassay (Innofluor Topiramate Assay System, OXIS International, Portland, OR, USA). LTG, VGB and GBP levels in serum and tissue extracts were analysed by established HPLC methods (Kilpatrick et al, 1996; Forrest et al, 1996; Ratnaraj and Patsalos, 1998).

2.3.5 Statistical analysis

Group results were expressed as mean values ± standard error of the mean. Drug concentrations between *mdr1a* (-/-) and *mdr1a* (+/+) mice were compared using Student's

two-sample t-test. Statistical calculations were made with use of Minitab for Windows software (version 11.21).

2.4 Results

Serum and tissue concentrations of the new AEDs are shown in Figures 19-25. Results for VPA were not available due to technical problems with the assay.

Compared with wild-type mice, *mdr1a* (-/-) mice had significantly higher brain concentrations of TPM at 30 minutes (p=0.04) and 60 minutes (p=0.01) after subcutaneous injection (Figure 23). PHT reached a higher level in the brains of *mdr1a* (-/-) mice at 240 minutes after injection (p=0.04) compared to *mdr1a* (+/+) mice (Figure 20). CBZ (Figure 21) concentrations were also higher in the brains of *mdr1a* (-/-) mice at 240 minutes after injection, although the difference failed to reach statistical significance (p=0.07). There was no difference in brain concentrations of PB, LTG, GBP or VGB between *mdr1a* (-/-) mice and *mdr1a* (+/+) mice at any time points investigated.

Compare to wild-type mice, CBZ (Figure 21) concentrations were higher in the liver of *mdr1a* (-/-) mice at 240 minutes after injection (p=0.02). However, liver concentrations of PHT (Figure 20) at 240 minutes (p=0.02) and of GBP (Figure 24) at 30 minutes (p=0.03) after injection were lower in the *mdr1a* (-/-) mice. For all eight drugs, there was no difference in concentrations in serum, heart or skeletal muscles between the two genotypes.

2.5 Discussion

This study was designed to examine whether P-gp at the BBB limits entry of AEDs into the brain. Drugs were administered by subcutaneous injection to bypass intestinal

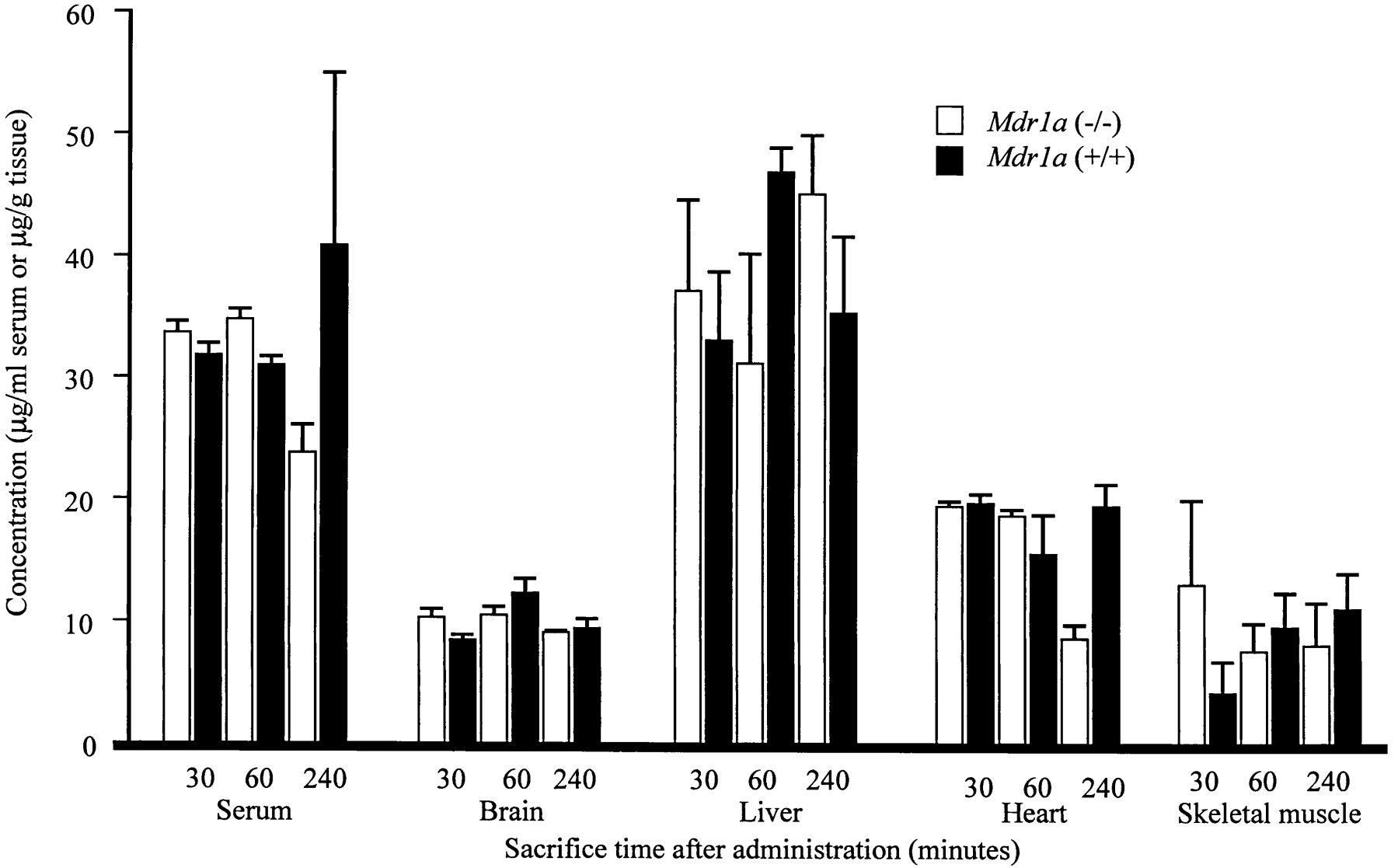


Figure 19. Concentrations of phenobarbital in *mdr1a* (-/-) mice and *mdr1a* (+/+) mice after subcutaneous injection of 20mg/kg. N=3 per group. Bars represent standard error.

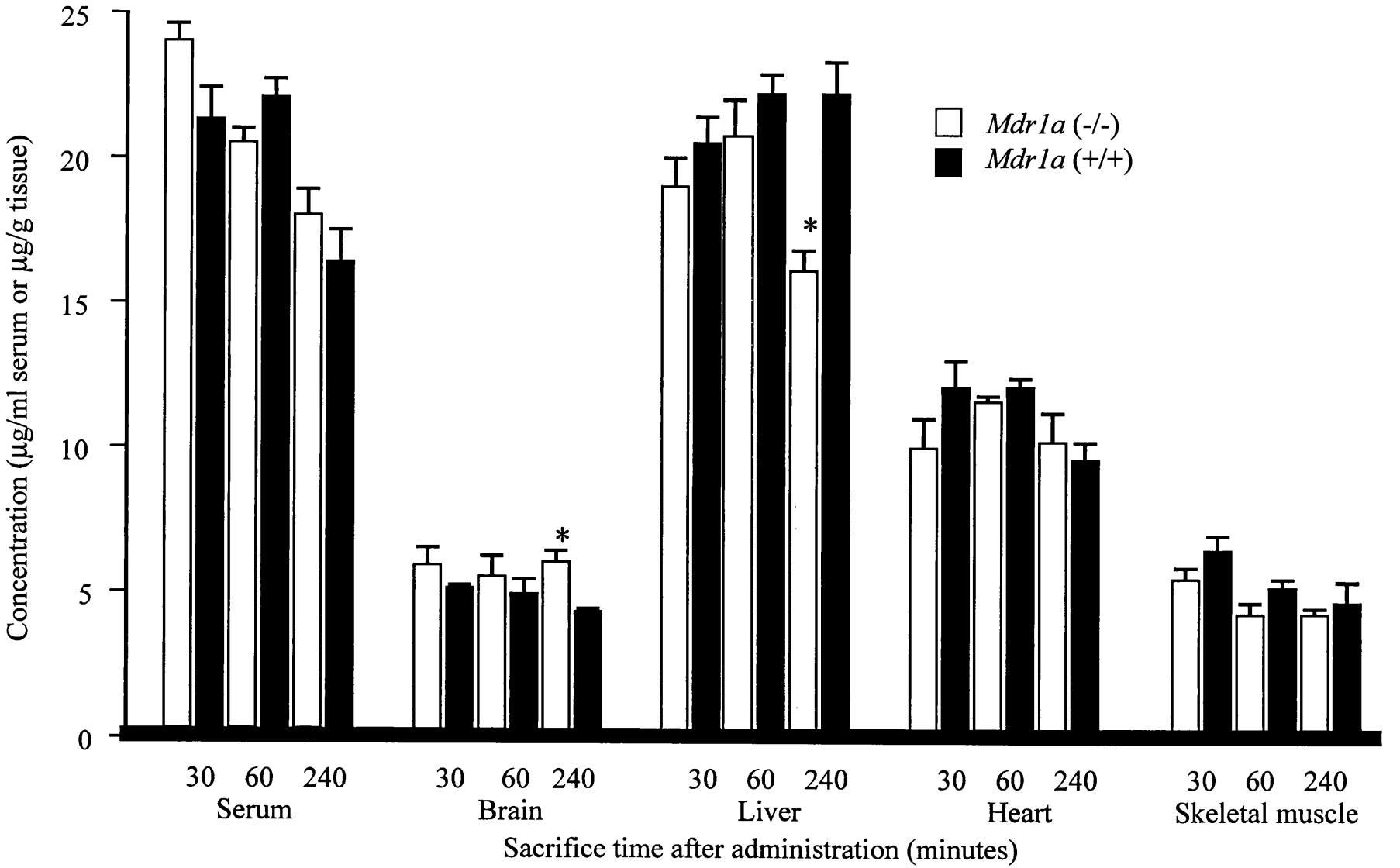


Figure 20. Concentrations of phenytoin in *mdr1a* (-/-) mice and *mdr1a* (+/+) mice after subcutaneous injection of 20mg/kg. N=3 per group. Bars represent standard error. *p=0.02 compared with *mdr1a* (+/+) mice.

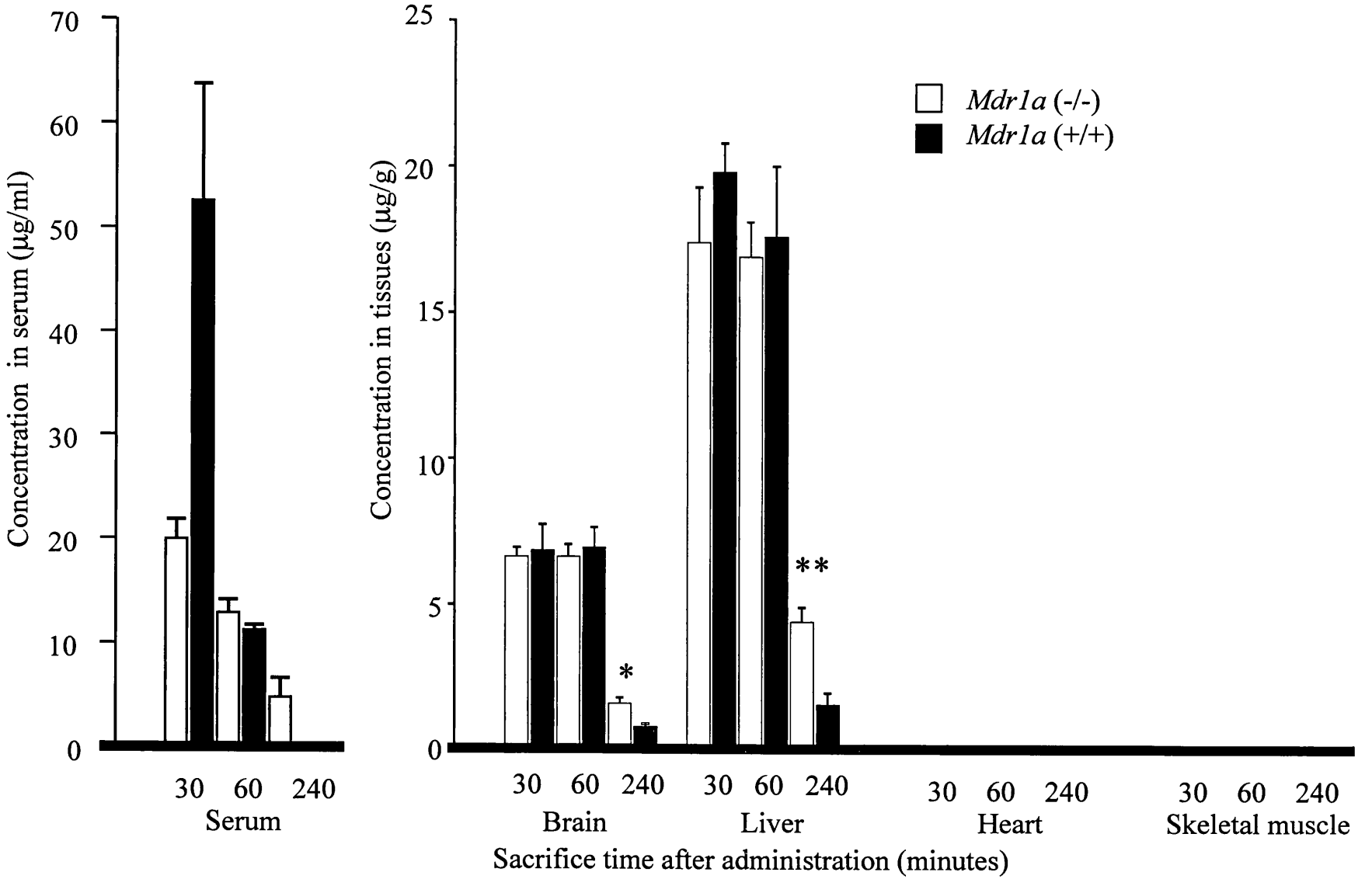


Figure 21. Concentrations of carbamazepine in *mdr1a* (-/-) mice and *mdr1a* (+/+) mice after subcutaneous injection of 20mg/kg. N=3 per group. Bars represent standard error. *p=0.07, **p=0.02 compared with *mdr1a* (+/+) mice.

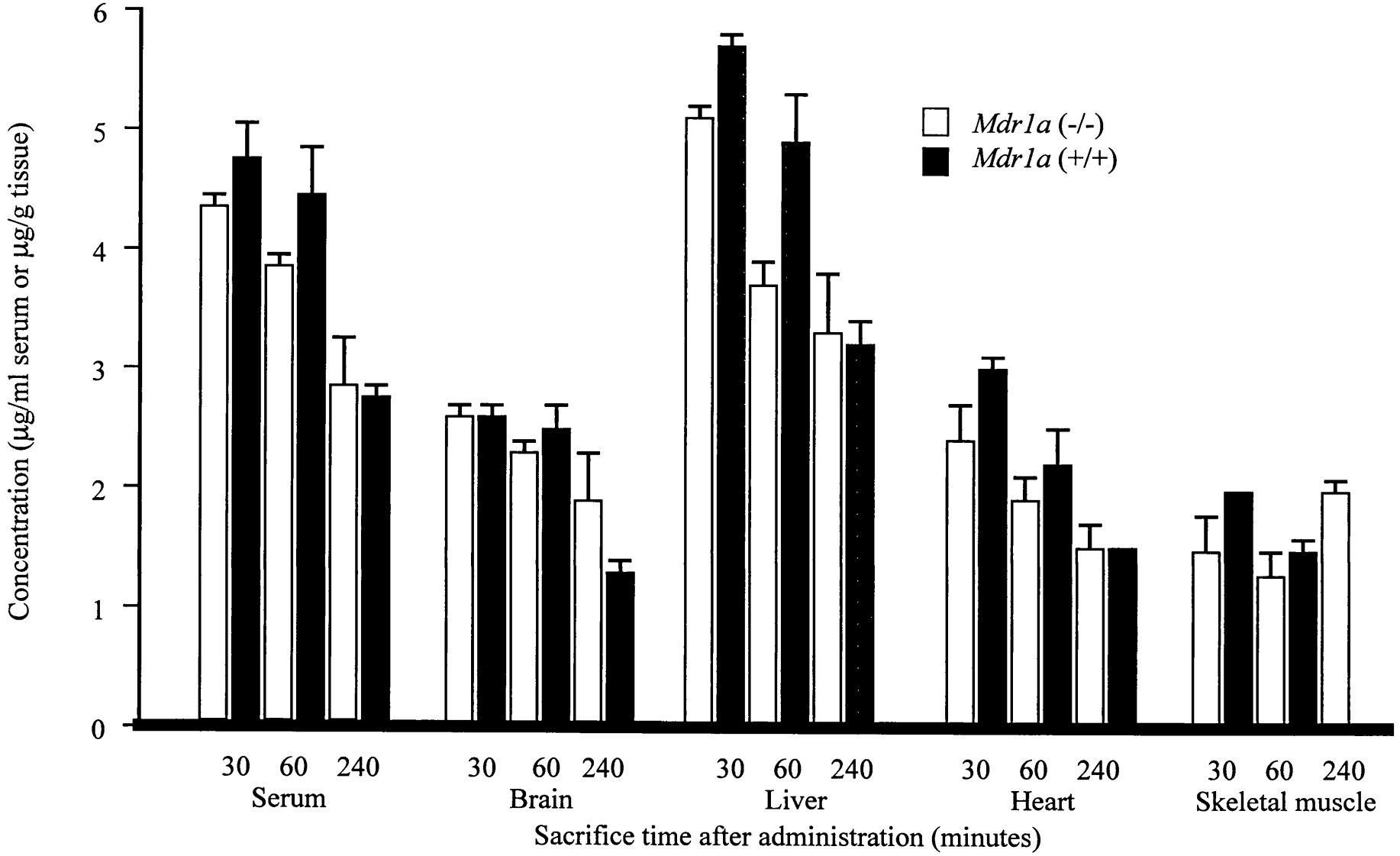


Figure 22. Concentrations of lamotrigine in *mdr1a* (-/-) mice and *mdr1a* (+/+) mice after subcutaneous injection of 5mg/kg. N=3 per group. Bars represent standard error.

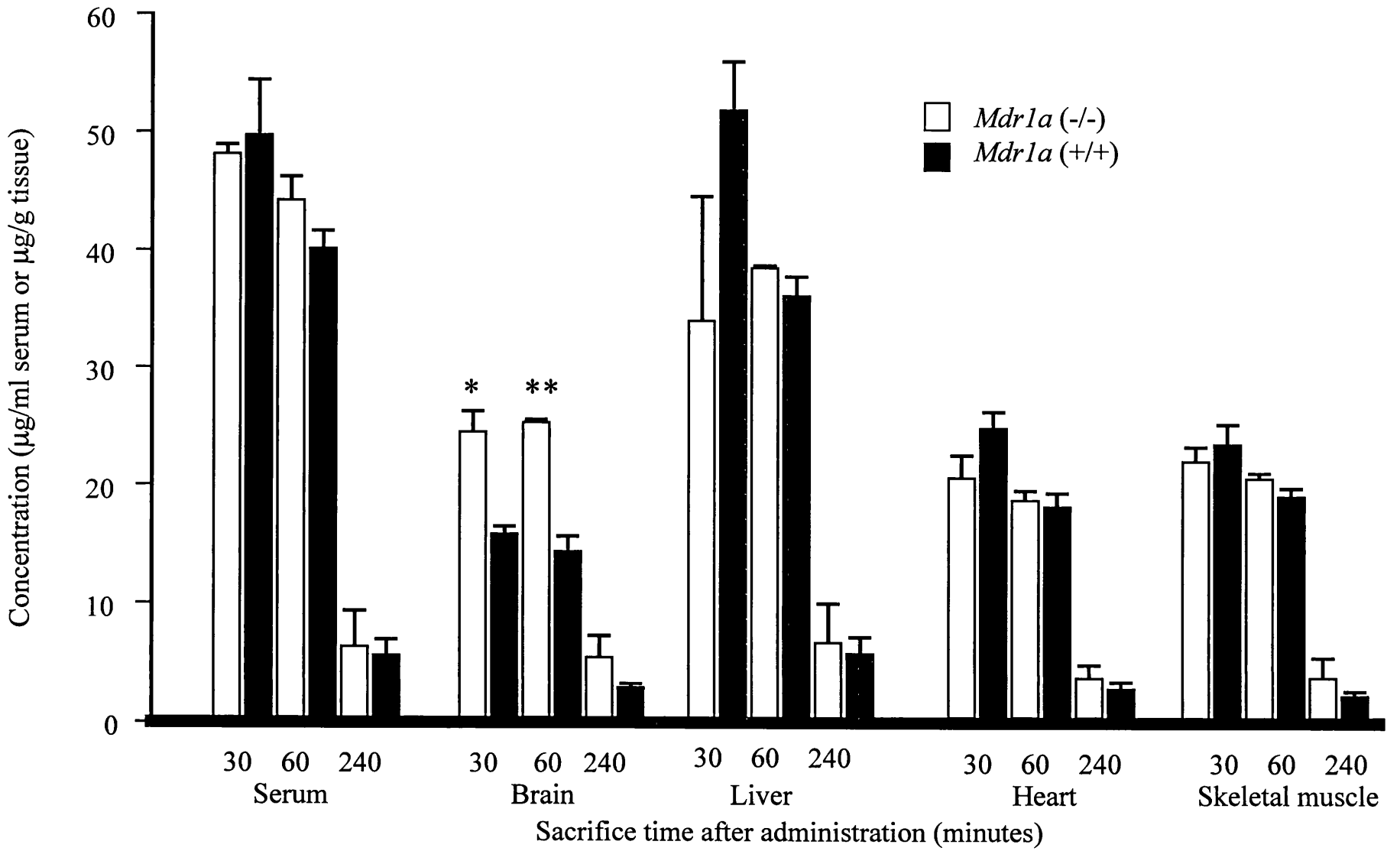


Figure 23. Concentrations of topiramate in *mdr1a* (-/-) mice and *mdr1a* (+/+) mice after subcutaneous injection of 50mg/kg. N=3 per group. Bars represent standard error. *p=0.04, **p=0.01 compared with *mdr1a* (+/+) mice.

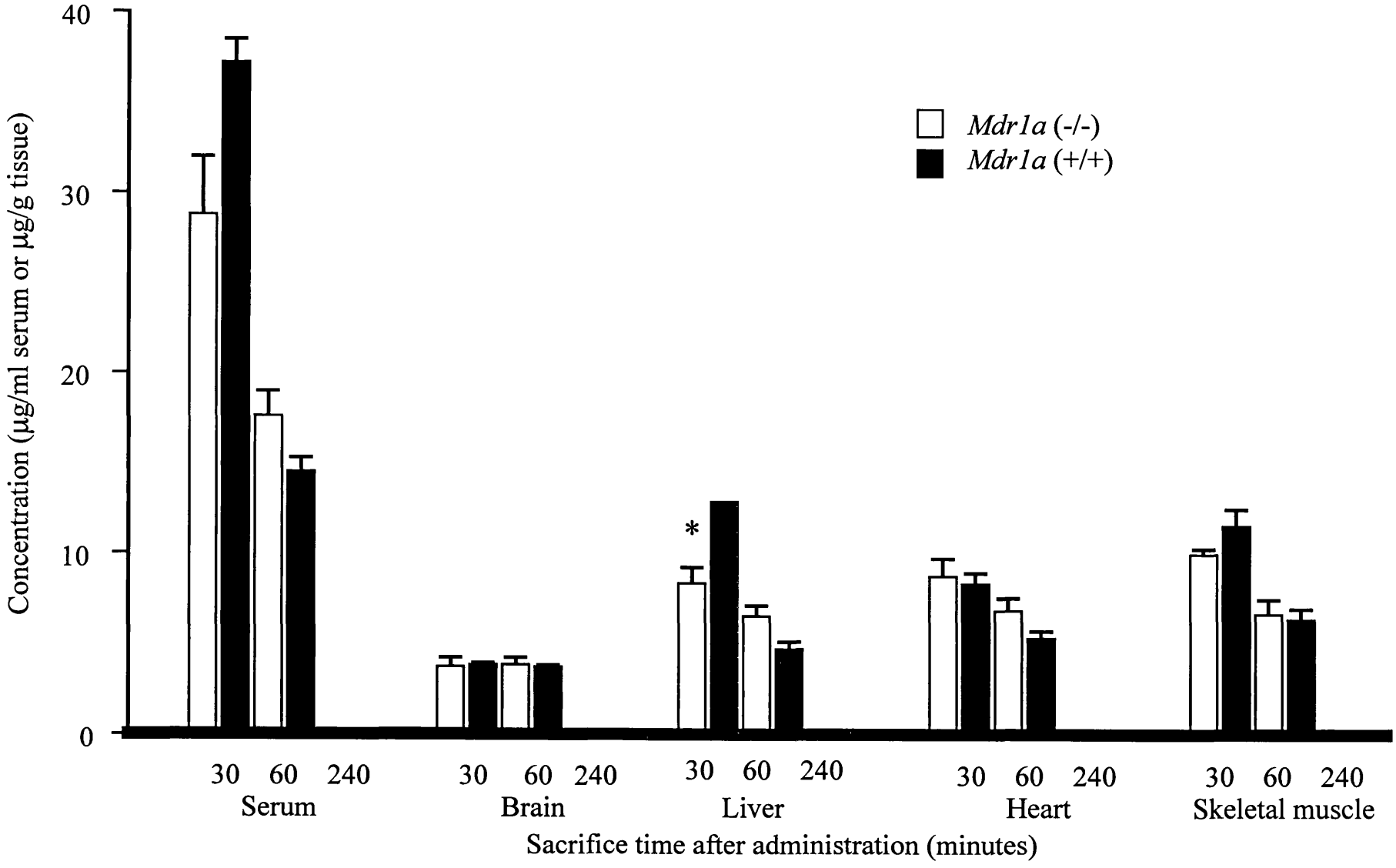


Figure 24. Concentrations of gabapentin in *mdr1a* (-/-) mice and *mdr1a* (+/+) mice after subcutaneous injection of 50mg/kg. N=3 per group. Bars represent standard error. *p=0.03 compared with *mdr1a* (+/+) mice.

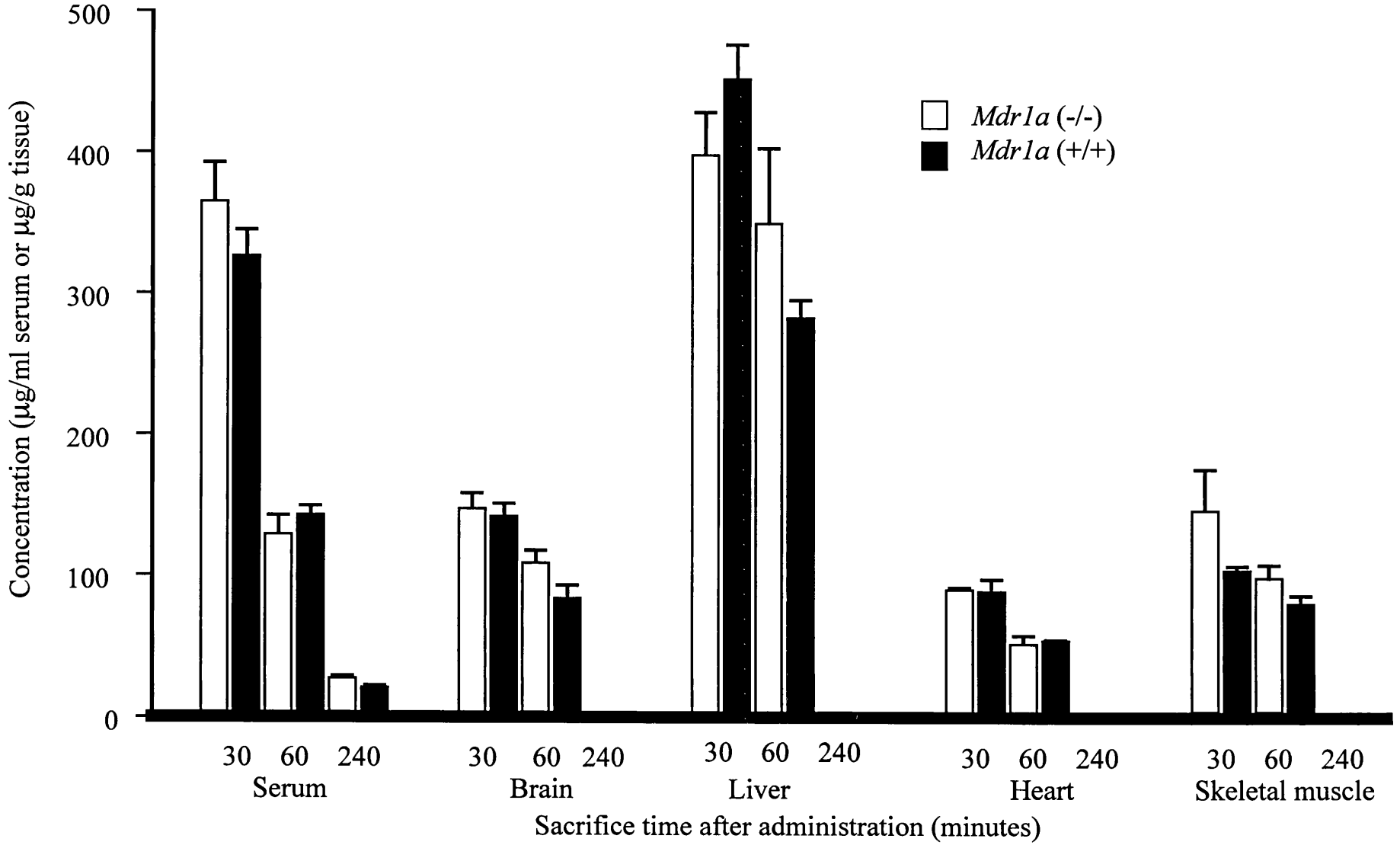


Figure 25. Concentrations of vigabatrin in *mdr1a* (-/-) mice and *mdr1a* (+/+) mice after subcutaneous injection of 500mg/kg. N=3 per group. Bars represent standard error.

absorption which might be increased in *mdr1a* (-/-) mice in the absence of gut P-gp (Sparreboom et al, 1994). Single administration was employed to avoid the effect of hepatic enzyme induction or inhibition during chronic dosing of some AEDs (Part I; Section 3.3.4). In some cases drug concentrations were below the lower quantifiable limit of the assays, particularly in cardiac and skeletal muscles where drug penetration tends to be low. Blood and tissue concentrations were undetectable at 240 minutes after injection of some AEDs, particularly those with short elimination half-lives, such as GBP and VGB.

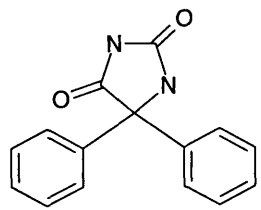
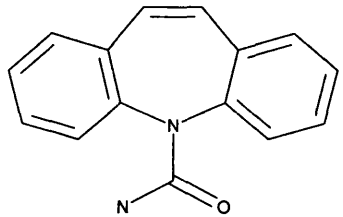
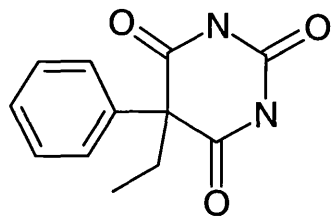
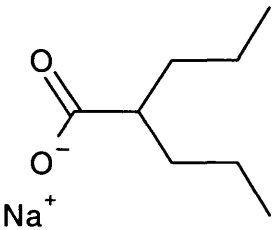
Higher brain levels of TPM, PHT, and possibly CBZ were found in the *mdr1a* (-/-) mice compared with *mdr1a* (+/+) mice, with no difference in serum levels, suggesting that these drugs are substrates of P-gp at the BBB. Maximal difference in drug concentration between the two genotypes was observed for TPM at 60 minutes after administration (78% higher in the knockout mice). Previous studies have reported up to 90-fold higher brain levels in the *mdr1a* (-/-) mice for certain substrates (Schinkel et al, 1994), but these studies tended to employ radiolabelled drugs, the measurement of which do not distinguish metabolites from the parent compound. With the exception of GBP and VGB which are largely excreted unchanged by the kidney (Wilson and Brodie, 1996), all the AEDs investigated in this study undergo extensive metabolism. Measuring the parent pharmacoactive moieties as opposed to radiolabelled compounds, therefore, reflects more truly their pharmacokinetic profiles.

Liver concentrations were significantly higher in *mdr1a* (-/-) mice than wild-type mice for CBZ, but lower for PHT and GBP. These differences need to be interpreted with caution. The liver is highly vascular and it was, therefore, not possible to completely remove excess blood from it. Homogenised liver samples could easily be contaminated by drug in the

vascular compartment, which is possibly the case for CBZ and GBP since differences in serum concentrations in the same direction between the two genetic types were observed. Interpretation is further complicated by the co-expression of *mdr1b* in the liver (Table 27). Compensatory upregulation of *mdr1b* in liver and kidney (but not the brain) has been reported in *mdr1a* (-/-) mice (Schinkel et al, 1994).

Comparison of the physical and chemical properties of the eight AEDs investigated might yield some clues as to why only TPM, PHT and CBZ were identified as P-gp substrates (Figure 26). Apart from hydrophobicity, a "typical" P-gp substrate is said to be large and positively charged at physiological pH (7.35-7.45) with a planar ring structure (Part III; Section 1.2.4). TPM is the heaviest (molecular weight 339) among the AEDs studied and the molecular weight of both PHT and CBZ are over 230. With pKa (the pH at which the compound is neutral) above 7.45, TPM and PHT are the only two that are positively charged at physiological pH. The two fused benzene rings afford CBZ a planar conformation (Faigle and Feldmann, 1995). Functionally, PHT and CBZ are both inducers of cytochrome P450 (CYP), which has been suggested to play a complementary role with P-gp in drug resistance (see Section 1.2.5). TPM increases the clearance of digoxin and the oestrogenic component of oral contraceptives (Garnett, 2000), also suggesting induction of CYP isozymes. However, PB, the archetypal CYP inducer, does not appear to be transported by *mdr1a* according to results of this study.

These data suggest that among the eight AEDs studied, TPM is the strongest P-gp substrate at the BBB. Differences in brain levels were observed at 30 minutes and 60 minutes after injection for TPM, but only at 240 minutes for PHT and CBZ. This may reflect the shorter

	Phenobarbital	**Phenytoin**	**Carbamazepine**	**Sodium valproate**
				
Molecular weight	232	252	236	166
Charge	Slightly negative	Positive	N/A	Negative

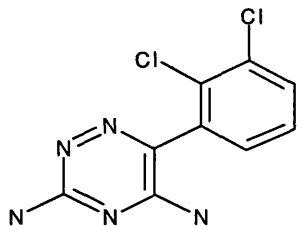
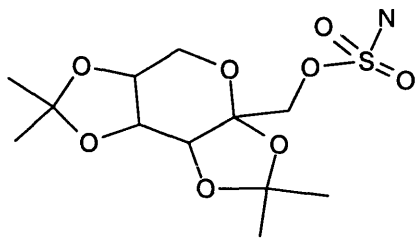
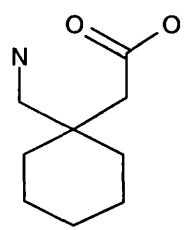
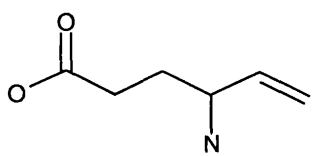
	Lamotrigine	**Topiramate**	**Gabapentin**	**Vigabatrin**
				
Molecular weight	256	339	171	129
Charge	Negative	Positive	Zwitterionic	Zwitterionic

Figure 26. Structures and chemical properties of the antiepileptic drugs investigated in *mdr1a*(-/-) and *mdr1a*(+/+) mice.

elimination half-life of TPM compared to the other two drugs observed clinically (Faigle and Feldmann, 1995; Trieman and Woodbury, 1995; Garnett, 2000).

PHT is the only AED that has been investigated previously for possible transport by P-gp. The finding of higher brain levels of PHT in *mdr1a* (-/-) mice than *mdr1a* (+/+) in our study provides *in vivo* evidence to support previous *in vitro* studies which have also identified PHT as a weak P-gp substrate (Tishler et al, 1995; Schinkel et al, 1996). An earlier study in *mdr1a* (-/-) mice found no difference in the tissue distribution of [^{14}C]PHT compared to wild-type mice (Schinkel et al, 1996), but the use of radiolabelled compound might not have reflected the drug's true pharmacokinetics. If the majority of the PHT metabolites (also radiolabelled) were not P-gp substrates and penetrated the brain easily, their presence might completely mask any potential differences for the parent drug.

These results also suggest CBZ to be a possible weak substrate of P-gp. Since the major metabolite of CBZ, 10,11-epoxide, also possesses antiepileptic activity, in hindsight, its tissue concentrations should have been analysed in addition to those of CBZ itself.

In conclusion, results from this pilot study suggest that TPM, PHT, and possibly CBZ are substrates of *mdr1a* P-gp. These findings need to be confirmed in larger scale experiments. A larger number of animals analysed across a wider range of doses and time points would allow more detailed profiling of the drugs' pharmacokinetics. The effect of chronic dosing should also be investigated to mimic their clinical usage more closely, and to examine whether these AEDs, similar to many other substrates, also induce the expression of P-gp. Since experiments in rats suggest that *mdr1b* is only expressed in the hippocampus in the brain (data presented in Part III, Section 4), hippocampal concentrations of AEDs may be

compared between *mdr1b* (-/-) mice and *mdr1b* (+/+) mice to investigate whether they are substrates of *mdr1b* P-gp. Finally, studies to determine whether co-administration of substrate AEDs with a P-gp inhibitor may enhance their access to the brain would aid assessment of the feasibility of such "rational polytherapy" in the clinical setting.

3 Recurrent materials and methods for the quantification of *MDR1* expression

3.1 Quantitative reverse transcriptase-ploymerase chain reaction – the theories

As discussed in Part III; Sections 1.2.7 and 1.2.8, the control of *MDR1* expression is at the transcriptional level and the amount of mRNA is generally regarded as a reliable reflection of the level of gene expression. A number of procedures are widely employed for determining the abundance of a particular mRNA in a sample of total RNA, including northern blot, slot-blot analysis, in situ hybridisation, RNAase protection assay, and reverse transcriptase-polymerase chain reaction (RT-PCR). Since the subsequent experiments (Part III, Sections 4 to 7) involved analysis of small discrete regions of rat brain in which the level of *mdr1* expression had not been previously studied, an assay that is both selective and sensitive was required. In addition, clinical epileptic brain tissues acquired during resective surgery (Part III, Section 8) can be poorly preserved, thus some tolerance to degradation was also necessary.

3.1.1 Drawbacks of common techniques to measure mRNA

Northern analysis is straightforward and versatile, but is very vulnerable to RNA degradation and relatively insensitive. Although RNAase protection assay is more sensitive than northern analysis, it still requires μg quantities of RNA and is subject to RNA degradation. Slot-blotting is prone to error through nonspecific hybridisation. A further common problem with these assays is the need to compare the results with a control as they generate an arbitrary number. In order to correct for assay variations, it is also necessary to compare the signal from the assay RNA to that of another gene whose expression is assumed to be unchanged in the experimental system. Such an assumption might not be valid in this case.

3.1.2 RT-PCR

In RT-PCR, a retroviral reverse transcriptase enzyme, e.g. Moloney-murine leukaemia virus (MMLV) or Avian Myoblastosis virus (AMV), uses random hexamers or oligo deoxythymidine [oligo$(dT)_{15}$] to prime the synthesis of cDNA transcripts from mRNA. The cDNA is then specifically amplified exponentially using sequence specific oligonucleotide primers in the PCR. Compared to AMV, MMLV has higher stability and lower intrinsic RNase H activity, while the use of random hexamers, unlike oligo(dT) primers, has the flexibility of acting on mRNA without a poly$(A)^+$ tail (Promega, 1996).

RT-PCR can detect the RNA transcript irrespective of the paucity of starting material or relative abundance of the specific mRNA. Compared to other RNA detection techniques, RT-PCR is specific, sensitive, and tolerant to low level RNA degradation and suitable for use on clinical and other small tissue samples (Noonan et al, 1990; Tishler et al, 1995; Zhang et al, 1996a; 1996b). In addition, when combined with a competitive internal standard, RT-PCR generates an absolute quantification without reference to a control sample or comparison gene, making it suitable for ongoing day-to-day analysis (Zhang et al, 1996a).

3.1.3 Competitive quantitative RT-PCR

Theoretically, the abundance of an individual mRNA transcript can be determined by knowledge of the amount of total RNA used in the initial cDNA synthesis reaction, the amount of cDNA used in the PCR reaction, and the number of PCR cycles necessary to generate sufficient detectable products. In practice, it is much more complicated. A major drawback to exponential amplification is that small sample to sample differences in amplification translate to large differences in product yield. In addition, there may be

confounding factors influencing the final amount of the detectable product, such as temperature variation across the thermal cycler block resulting in variable reaction efficiencies, or even variation in loading the final products to the agarose gel which is often not prepared with uniform quality. Accurate determination of the absolute abundance of a specific transcript by RT-PCR requires the use of competitive RT-PCR techniques.

Competitive quantitative RT-PCR uses a single set of primers to amplify both the target mRNA of interest and an exogenously added competitor RNA (internal standard) of known concentration, but is distinguishable from the gene of interest. A serial dilution of the internal standard is added to replica samples of tissue RNA in amounts that span the target mRNA level at the reaction outset (the RT step). Any confounding variations between samples discussed above are thus accounted for. At the concentration of internal standard where the yield of competitor product matches that of the endogenous target, the two templates are presumed to have been present in equal amounts at the start of the reaction. Competitive RT-PCR requires the construction of a template as internal standard which undergoes RT and PCR at the same efficiency as the target mRNA. A method of discriminating the products from each other is also required. The internal standard usually consists of the target mRNA with a small, but significant, insertion or deletion in order that its RT-PCR products can be separated electrophoretically from those of the target mRNA on an agarose gel.

The major disadvantage of the use of competitive RT-PCR to quantify target mRNA is that it is technically laborious and costly, as multiple RT and PCRs are required for each sample. Nevertheless, its superior specificity and sensitivity makes it the technique-of-choice for quantifying *mdr1* mRNA in the experimental models described in this thesis.

3.2 Isolation of total RNA

Solutions used in RNA analysis were treated with 0.01% v/v diethylpyrocarbonate (DEPC) overnight at room temperature to inactivate ribonucleases (RNases), then autoclaved to remove any remaining DEPC. Sterile, disposable deoxyribonuclease (DNase)- and RNase-free plasticware was used throughout. All re-used reaction tubes or instruments in direct contact with tissues were washed thoroughly in 3% v/v hydrogen peroxide followed by 0.1% DEPC treated distilled water (dH2O). To avoid cross contamination all pipetting was carried out using DNase- and RNase-free filter tips and a fresh aliquot of water was used each time. Reagents and tissues were kept on ice at all times during manipulation to prevent degradation of RNA.

Total RNA was extracted from brain tissues using a commercial kit (RNAgent Total RNA Isolation System, Promega, Southampton, UK). The volumes of reagents used were adjusted according to the weight of the tissue as recommended in the protocol. Brain tissue was dropped into ice cold denaturing solution (1.2ml per 100µg tissue) supplied with the kit and homogenised using a Kinematica polytron homogeniser (Philip Harris Scientific, Aberdeen, UK) on maximum setting for 30 seconds. A 600µl aliquot of the homogenised solution was removed into a clean 2ml microtube, while the remainder was stored at –80°C for future use if required. A 60µl aliquot of sodium acetate (2M; pH 4.0) was added to the microtube and mixed thoroughly by inverting the tube 4-5 times. Phenol:chloroform:isoamyl alcohol (125:24:1; 600µl) was removed from the lower organic phase of the bottle supplied and added to the tissue sample tube. The mixture was shaken vigorously for 10-15 seconds and left to chill on ice for 15 minutes. The mixture was centrifuged at 10,000x*g* for 20 minutes at 4°C in a pre-chilled centrifuge (Heraeus

Sepatech). The top aqueous layer was carefully removed to a fresh tube, avoiding the interface, which contains genomic DNA. An equal volume of isopropanol was added, the sample was inverted several times to mix and finally stored at -20°C for 3 to 6 hours to precipitate the RNA.

The RNA was pelleted by centrifuging the sample at 10,000x*g* for 20 minutes at 4°C. The supernatant was poured off and the pellet washed in 75:25 ethanol/DEPC treated dH_2O. The sample was centrifuged again at 10,000x*g* for 20 minutes at 4°C. The supernatant was aspirated and the pellet allowed to dry on ice for 15 minutes. The RNA pellet was resuspended in 15-20µl DEPC-treated dH_2O. The total RNA concentration was determined by measuring the OD_{260}, in duplicate, on a spectrophotometer (UV1101 Biotech Photometer, WPA, Cambridge, UK), where 1 optical density unit (OD) corresponds to approximately 40µg RNA/ml. The purity of the RNA was determined by the ratio of OD_{260}/OD_{280}, where pure RNA has a ratio of 2.0 (Sambrook et al, 1989). In practice, ratios of between 1.7 and 2.2 were considered acceptable. The integrity of the extracted RNA was confirmed by running 300ng on an ethidium bromide-stained 1% w/v agarose gel. The 28S and 18S and ribosomal RNA bands were identified under ultraviolet light and the RNA was stored at –80°C until required. The procedures of gel preparation and electrophoresis are described in Part III; Section 3.5.3.

3.3 Removal of contaminating DNA

Genomic DNA contamination of the RNA would interfere with the subsequent RT-PCR quantification as DNA absorbs light at the same wavelength as RNA. In addition, genomic DNA would compete with the target and internal standard cDNA during PCR. To remove contaminating genomic DNA, RNA was treated with a DNase (RQ1 deoxyribonuclease,

Promega, Southampton, UK) which digests double stranded DNA but has no RNase activity, thus leaving the RNA intact.

The reaction mixture was as follows:-

RNA (200ng/µl)	100µl
$MgCl_2$ (25mM; Promega)	40µl
10x Mg^{2+} free PCR buffer (Promega)	20µl
RQ1 DNase (1U/µl)	40µl
Total volume	200µl

The mixture was incubated at 37°C for 1 hour and the reaction terminated by adding an equal volume (200µl) of phenol/chloroform, vortexing for 10 seconds, then centrifuging at 10,000x*g* for 5 minutes at 4°C in a pre-chilled centrifuge. The upper aqueous layer was removed into a clean tube and an equal volume of chloroform was added. The sample was vortexed for 10 seconds and then centrifuged at 10,000x*g* for 5 minutes at 4°C. The upper aqueous layer was again removed into a clean tube and 3 volumes of 100% ethanol and 0.1 volumes of DEPC treated sodium acetate (3M; pH 5.5) was added. The tube was inverted several times and stored at -20°C for 3 hours to precipitate the RNA. Thereafter, the sample was centrifuged at 10,000xg for 30 minutes at 4°C. The supernatant was poured off and the pellet washed in 75:25 ethanol/ DEPC treated dH_2O. It was centrifuged again at 10,000xg for 30 minutes at 4°C. The supernatant was aspirated and the pellet dried on ice for 15 minutes. The RNA pellet was re-dissolved in 10µl DEPC-treated dH_2O. The concentration, purity, and integrity were determined by spectrophotometry and agarose gel electrophoresis as described above. The RNA was stored at -80°C until required.

3.4 Preparation of internal standard control RNA

Internal standard control (ISC) RNAs were prepared from DNA plasmids as previously described (Zhang et al, 1996a). The same protocol was used for the internal standards of rat *mdr1a* and *mdr1b*, and human *MDR1*. Aliquots of DNA plasmids of rat *mdr1a* and *mdr1b* and human *MDR1* ISC were kindly gifted by Dr T.W. Gant (MRC Toxicology Unit, Leicester, UK). They were constructed by insertion of an extra sequence into the corresponding genes (Zhang et al, 1996a). The RT-PCR products of an ISC were, therefore, visualised as a heavier band than those of the tissue RNA on the agarose gel.

3.4.1 Transformation of competent cells with ISC plasmid DNA

A commercial kit (AdvanTAge PCR Cloning Kit, CLONTECH Laboratories UK Ltd, Basingstoke, UK) was used to transform competent cells with ISC plamids. For each plasmid, a tube of frozen TOP10F' *E. coli* cells and β-mercaptoethanol were thawed on ice and SOC medium was allowed to defrost to room temperature. A 2μl aliquot of β-mercaptoethanol was pipetted into the cells and stirred gently. Thereafter, 2μl of plasmid was added and the mixture was again stirred gently. The mixture was kept on ice for 30 minutes before heating to 42°C for exactly 30 seconds in a water bath. The mixture was left on ice for a further 2 minutes. A 250μl aliquot of SOC medium was added and the mixture incubated in a shaking incubator (37°C) at 225 r.p.m. for 1 hour. Therefore, 50μl of mixture was spread onto an L-amp agar plate (Appendix 2) and 200μl to another plate. The plates were left upright at room temperature for 30 minutes, then inverted and incubated at 37°C overnight. Normal TOP10F' cells are sensitive to the ampicillin in the plate and, therefore, do not grow. *MDR* ISC plasmids confer ampicillin resistance, thereby allowing transformed cells to multiply into colonies.

3.4.2 Large scale preparation and purification of ISC plasmid DNA

Large quantities of the transformed cells were grown and the plasmid isolated by alkali lysis as described (Sambrook et al, 1989). The plasmid was then purified using a commercial kit (QIAfilter Maxi, QIAGEN, Crawley, UK).

Using a sterile pipette tip, one of the colonies containing the plasmid was picked from the L-amp agar plate and dropped into 2ml LB-amp medium (Appendix 2) in a 25ml sterile tube. The culture was placed in a shaking incubator (37°C) at 225 r.p.m. overnight. The culture plate was discarded.

A 500µl aliquot of the overnight culture was added to 250ml freshly prepared LB-medium. This was again incubated (37°C) at 225 r.p.m. overnight. A glycerol stock of the transformed cells was prepared by adding 150µl of the culture solution to 850µl 100% glycerol (autoclaved), which was stored at –70°C and from which colonies could be re-grown by re-spreading on an agar plate if required.

The following day the culture medium was poured into a clean bottle and centrifuged at 6000x*g* for 15 minutes at 4°C. The supernatant was decanted and the bacterial pellet resuspended completely in 10ml Buffer P1 provided in the kit (resuspension buffer). A 10ml aliquot of Buffer P2 (lysis buffer) was added, mixed gently by inverting 4-6 times, and incubated at room temperature for exactly 5 minutes. A 10ml aliquot of Buffer C (neutralisation buffer) was added to the lysate and mixed by inverting 4-6 times. The lysate was poured into the barrel of the QIAfilter cartridge immediately and left to incubate at room temperature for 10 minutes. During this period of time, a QIAGEN-tip 500

column was equilibrated by applying 10ml Buffer QBT, which was allowed to pass through the resin in the column by gravity flow. The cap was removed from the cartridge nozzle and the plunger was inserted into the cartridge to filter the cell lysate. The filtered lysate was then allowed to enter the previously equilibrated resin by gravity flow. The resin column was washed twice with 30ml Buffer QC; again the wash buffer was allowed to move through the resin by gravity flow. A 15ml aliquot Buffer QF was poured into the resin column to elute the DNA, which was collected in a clean 30ml polypropylene tube. DNA was precipitated by adding 10.5ml isopropanol (room temperature). The tube was inverted several times and centrifuged at 15,000x*g* at 4°C for 30 minutes. The supernatant was decanted and the DNA pellet washed with 5ml 70% ethanol (room temperature) and centrifuged again at 15,000xg for 10 minutes. The resulting supernatant was carefully decanted. After air-drying the pellet for 10 minutes, the DNA was redissolved in 250µl TE buffer (pH 8.0). The concentration of the DNA plasmid was determined by measuring the OD_{260} on a spectrophotometer (UV1101 Biotech Photometer, WPA, Cambridge, UK), where 1 absorbance unit is equivalent to 50µg DNA/ml.

3.4.3 Linearisation of plasmid DNA

Each of the rat *mdr1a*, *mdr1b* and human *MDR1* ISC plasmids was linearised as follows:-

Plasmid DNA (10µg)	x µl (where x is concentration-dependent)
10x Buffer	5µl
Restriction enzyme (10U/ml)	10µl
DEPC-treated dH_2O	y µl (where y is dependent on x)
Total volume	50µl

MDR1 was linearised with *PstI* (Buffer H), and *mdr1a* and *mdr1b* with *NcoI* (Buffer D). The reaction was incubated at 37°C for 4 hours. The quality of the DNA was assessed and the absence of RNA confirmed by running 2µl of the digest mix on a 1% agarose gel with ~500ng of uncut plasmid in an adjacent lane to ensure that the digest was complete. The uncut plasmids, due to supercoiling, were visible as several bands of various lengths, while only single bands were seen with linearised plasmids.

3.4.4 Purification of linearised plasmid DNA

Each linearised plasmid DNA was purified using a commercial clean-up kit (Wizard DNA Clean-up System, Promega, Southampton, UK). A 40µl aliquot of DEPC-treated dH_2O was added to 10µl (~2µg) of the linearised plasmid mix. Thereafter, 1ml of Clean-Up resin was added and mixed by inversion. The resin/DNA mix was pipetted into the supplied syringe barrel. Vacuum was applied using a manifold (Vac-Man Jr. Laboratory Vacuum Manifold, Promega) to draw the solution through the minicolumn supplied. To wash the column, 2 ml of 80% isopropanol (v/v) was added to the syringe barrel and drawn through the minicolumn by vacuum. The resin was dried by applying vacuum for a further 30 seconds after the solution had been pulled through the column. The minicolumn was centrifuged at 10,000x*g* for 2 minutes to remove any residual isopropanol. The minicolumn was then transferred to a new microcentrifuge tube. A 50µl aliquot of prewarmed (65-70°C) DEPC-treated dH_2O was applied to the Minicolumn. After 1 minute, the minicolumn was centrifuged at 10,000x*g* for 20 seconds to elute the bound DNA.

3.4.5 In vitro transcription of ISC RNA

A 25µl aliquot of the purified DNA was transcribed by SP6 RNA polymerase as follows:-

5x transcription buffer	14µl
100mM dithiothreitol	7µl
Rnasin (40U/ml)	2µl
rNTP mix (2.5mM)	20µl
ISC DNA template	25µl
SP6 RNA polymerase (20U/ml)	2µl
Total volume	70µl

The reaction was incubated at 37°C for 1 hour. A 2µl (2U) aliquot of RNase-free RQ1 DNase (Promega) was then added and the incubation continued for a further 30 minutes to remove the DNA template. Thereafter, 80µl DEPC-treated dH_2O was added, followed by 150µl of phenol/chloroform and the mixture vortexed for 10 seconds. It was then centrifuged at 10,000x*g* for 5 minutes at 4°C. The upper aqueous layer was removed to which an equal volume of chloroform was added. The mixture was again vortexed for 10 seconds and centrifuged at 10,000x*g* for 5 minutes at 4°C. The upper layer was again removed, to which 3 volumes of ethanol and 0.1 volumes of DEPC treated sodium acetate (3M; pH 5.5) was added. The tube was inverted several times and stored at -20°C overnight. The next day the tube was centrifuged at 10,000x*g* for 30 minutes at 4°C. The supernatant was decanted and the pellet washed in 75:25 ethanol/ DEPC dH_2O. It was centrifuged again at 10,000x*g* for 30 minutes at 4°C. The supernatant was aspirated and the pellet dried on ice for 15 minutes. The RNA pellet was redissolved in 10µl DEPC-treated dH_2O and the concentration and purity was determined by spectrophotometry as described above.

A 1μg aliquot of the transcribed RNA was passed through a purification column (NucTrap probe Purification Column, Stratagene, Amsterdam, The Netherlands) to remove the unincorporated nucleotides following the manufacturer's instructions. The purified ISC RNA retrieved was quantified on a spectrophotometer as described above. To avoid degradation through thawing and re-freezing, dilutions of ISC RNAs were prepared fresh on the day of each RT-PCR experiment from an aliquot of the stock solution. Unused dilutions were discarded.

3.5 Reverse transcriptase-polymerase chain reaction

The methodology of qualitative and quantitative RT-PCR closely followed that developed by Zhang and colleagues (1996a), using the same primer sequences, reaction compositions and conditions. Each set of primers was designed such that they spanned an intron. This ensured that any products arising from amplification of contaminating genomic DNA (containing introns) would be longer than those from the RNA (without introns) and could be differentiated by electrophoretic separation. Primers were chosen from regions that bore the least homology amongst the genes to ensure specificity (Zhang et al, 1996a). The sequences of the primers, expected product size for rat *mdr1a, 1b* and human *MDR1*, and the molecular weight of the ISCs are given in Table 28.

3.5.1 Reverse transcription

For each sample of rat brain RNA, qualitative RT-PCR for both *mdr1a* and *mdr1b* was performed. RT-PCR for *MDR1* was performed for human tissues. Individual reaction mixtures consisted of the following:

Table 28. Primers used for reverse transcriptase-polymerase chain reaction (RT-PCR) of rat *mdr1a* and *mdr1b* and human *MDR1* and expected size of the RT-PCR products (derived from Zhang et al, 1996).

Gene		Primers	Nucleotides from translation start site	Expected product size (bp)		Molecular weight of ISC
				Tissue	ISC	
Rat *mdr1a*	Sense	5'-GATGGAATTGATAATGTGGACA-3'	1912-1933	351	749	153004.8
	Antisense	5'-AAGGATCAGGAACAATAAA-3'	2244-2262			
Rat *mdr1b*	Sense	5'-GAAATAATGCTTATGAATCCCAAAG-3'	1910-1934	326	721	146499
	Antisense	5'-GGTTTCATGGTCGTCGTCTCTTGA-3'	2212-2235			
Human *MDR1*	Sense	5'-AAAAAGATCAACTCGTAGGAGTG-3'	1992-2014	162	212	90851.4
	Antisense	5'-GCACAAAATACACCAACAA-3'	2135-2153			

ISC = internal standard control, bp = base pair

	µl
10x Mg^{2+}-free PCR buffer	1.00
$MgCl_2$ (50mM)	0.50
dNTP mix (100mM)	0.40
Random hexamers (90OD/ml)	0.10
Dithiothreitol (100mM)	0.10
Rnasin (40U/ml)	0.25
MMLV RT(200U/µl)	0.50
Tissue RNA (100ng/µl)	1.00
dH_2O (DEPC treated)	6.15
Total volume	10.00

In practice, since multiple reactions were carried out at any one time, a reaction mixture containing the reagents was prepared on the day of the experiment. A 9 µl aliquot of the mixture was pipetted into each reaction tube, to which 1µl of tissue RNA was added. The reaction was carried out in a thermal cycler (Perkin Elmer DNA Thermal Cycler 480, PE Biosystems, Norwalk CT, USA):

Hexamer annealing	23°C	10 minutes
Product extension	42°C	45 minutes
Reaction termination	99°C	10 minutes
Inactivation of RT	4°C	stand (at least 10 minutes)

3.5.2 Polymerase chain reaction

All the cDNA produced in the RT was used for the subsequent PCR which contained:

	µL
10x Mg^{2+}-free PCR buffer	1.0
$MgCl_2$ (50mM)	0.5
Sense primer (4 pmol/µl)	1.0
Antisense primer (4 pmol/µl)	1.0
W-1 detergent (1%)	1.0
Taq DNA polymerase (5U/µl)	0.2
cDNA (from RT above)	10.0
dH_2O	5.3
Total volume	20.0

Again, in practice, since multiple reactions were carried out at any one time, a reaction mixture containing the reagents was prepared on the day of the experiment. A 10µl aliquot of the mixture was pipetted into each reaction tube. The reaction was carried out in a thermal cycler (Perkin Elmer DNA Thermal Cycler 480, PE Biosystems, Norwalk CT, USA):

Denaturation	95°C	5 minutes	⌐
Primer annealing	56°C	2 minutes	x 1 cycle
Primer extension	72°C	1 minute	⌙

95°C	1 minute	⎫
55°C	1 minute	⎬ x 28 cycles
72°C	1 minute	⎭
95°C	1 minute	⎫
55°C	2 minutes	⎬ x 1 cycle
72°C	5 minutes	⎭
4°C	stand	

3.5.3 Visualisation of PCR products

A 2% (w/v) agarose gel was prepared by adding 2g agarose (BioGene, Kimbolton, UK) to 100ml electrophoresis buffer (Appendix 2) which was heated to boiling in a volumetric flask. Approximately 3µl of ethidium bromide (10mg/ml) was added to the agarose solution and mixed thoroughly by swirling. The solution was poured onto an electrophoresis tray (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) and allowed to stand at room temperature to form a gel. "Wells" were formed by placing a "comb" on the tray during cooling. The tray containing the gel (without the comb) was then immersed in electrophoresis buffer in a horizontal tank (Bio-Rad). A 15µl aliquot of the products in each PCR tube was added to 5µl of DNA loading buffer (Appendix 2) and the mixture was loaded to a single well on the gel. Electrophoresis was carried out at 110V for 1.5 hours. The PCR products were visualised under ultraviolet light (see below).

3.5.4 Quantitative RT-PCR

If mRNA was detected by qualitative RT-PCR, quantitative RT-PCR was performed to measure the absolute level of gene expression. During the RT step, 1μl of internal standard RNA was added and the volume of dH_2O was reduced accordingly. For each sample of tissue RNA, 7 reverse transcription reactions were set up with increasing amount of internal standard RNA competing with the same amount of target RNA. For example,

Reaction	Target RNA (ng)	ISC RNA (pg)	RT enzyme
1	100	0.01	+
2	100	0.05	+
3	100	0.1	+
4	100	0.5	+
5	100	1.0	+
6	100	0	-
7	dH_2O (DEPC-treated)	0	+

Reactions 6 and 7 acted as negative controls. PCR and gel electrophoresis were then carried out as described above. The concentration range of ISC was chosen such that it spanned the target mRNA concentration in the tissue. This ensured that the ratios of band volume of ISC/target across the ISC dilutions spanned across 1 during subsequent image analysis (see below).

3.5.5 Calculation of target mRNA concentration

When electrophoresis was finished, the agarose gel was placed directly under a digital densitometer (GelDoc, Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) for scanning. A

typical image is shown in Figure 27a. The top band corresponded to the heavier ISC which travelled slower than the target (tissue) RNA due to the extra inserted sequence. The intensity (volume) of each individual band was measured against a local background using Multi-Analyst 1.1 software (Bio-Rad Laboratories Ltd). In the example shown in Figure 27a, the following results were yielded:

Lane	Band position	RNA	Amount of ISC in weight (pg)	Amount of ISC in molecules ($x10^6$)	Band volume	Ratio of ISC/target
1	Upper	ISC	0.01	0.04	26.84	0.05
	Lower	Target			1109.82	
2	Upper	ISC	0.05	0.20	171.31	0.20
	Lower	Target			868.49	
3	Upper	ISC	0.1	0.39	251.96	0.44
	Lower	Target			571.72	
4	Upper	ISC	0.5	1.97	888.19	1.80
	Lower	Target			492.85	
5	Upper	ISC	1	3.93	1850.41	4.69
	Lower	Target			394.66	

Knowledge of the molecular weight of the ISC (Table 28) allowed calculation of the number of ISC molecules employed in each reaction. A double-$\log_{10}$ plot of the amount of ISC RNA (x-axis) against the ratio of ISC intensity/target intensity (y-axis) yielded a straight line (Figure 27b). Only graphs with a correlation coefficient (r^2) of at least 0.95 were used to ensure validity of the results.

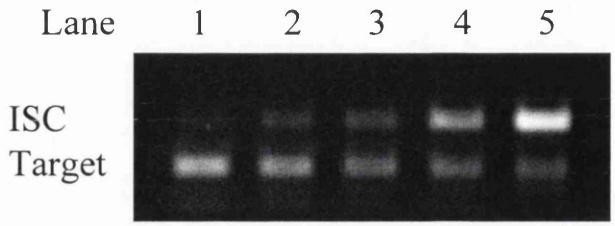


Figure 27a. Typical quantitative RT-PCR image after electrophoretic separation on an agarose gel. ISC=internal standard control

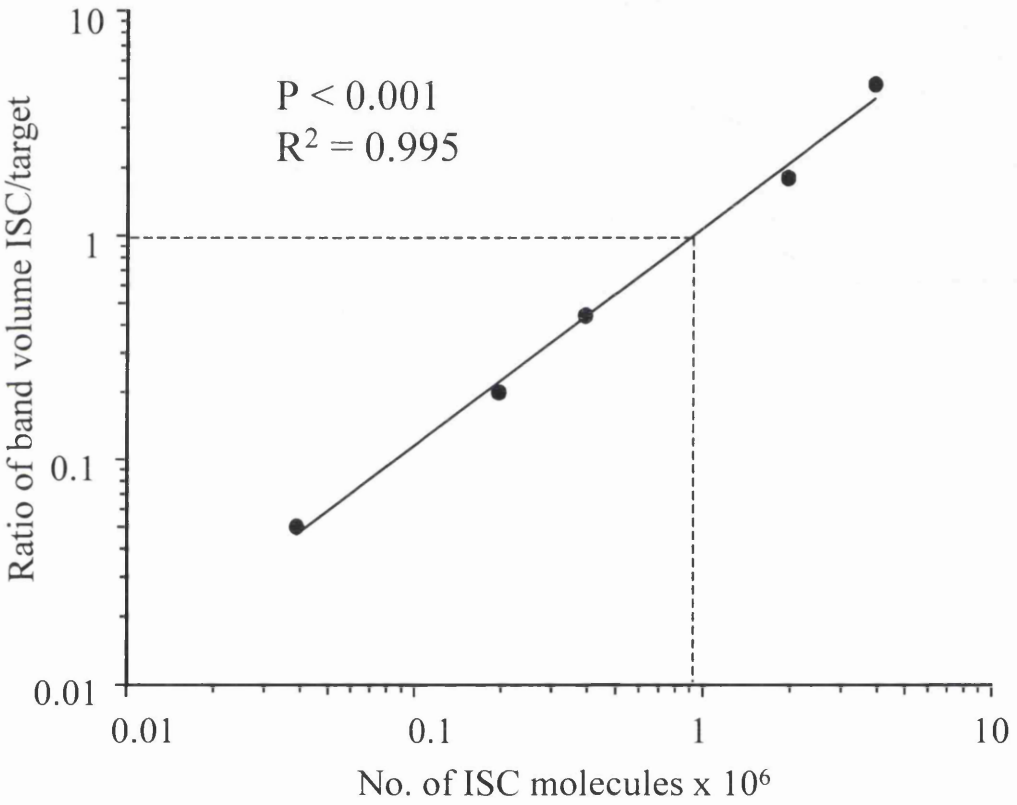


Figure 27b. Calculation of the number of target *mdr1* mRNA molecules by plotting the ratio of the band volume of internal standard control (ISC)/target against the known amount of ISC in the reactions.

When the ratio of internal standard intensity / target intensity = 1, the number of molecules of target mRNA is equivalent to that of ISC. The straight line equation could thus be used to calculate the absolute number of molecules of *mdr1* mRNA per ng of total RNA:

$$\mathbf{y = ax + b}$$

where **y** is the $\log_{10}$ ratio of band volume of ISC over that of target, **x** is the $\log_{10}$ number of molecules of ISC, **a** is the gradient of the slope and **b** is the y-axis intercept. In this example, the concentration of target *mdr1a* mRNA was 0.93×10^6 molecules per 100ng total RNA extracted from the tissue, or 9.3×10^3 molecules per ng total RNA. Statistical calculation was performed with use of Minitab for Windows (version 11.21).

4 Validation of RT-PCR assay and physiological expression of *mdr1* genes in rat brain

4.1 Introduction

Whereas the drug-transporting (Class I) P-gp is encoded by a single gene (*MDR1*) in man, two isoforms exist in rodents (Table 24a). Their tissue distribution in rats has not been well investigated. In mice, *mdr1a* and *mdr1b* P-gps have overlapping but distinct tissue distributions (Croop et al, 1989). Together they cover the same tissues as the single *MDR1* in man (Table 27), suggesting that they fulfil the same physiological role(s) in the different species (Schinkel, 1997).

4.1.1 Which isoform is expressed in which region?

Although genetically engineered mice deficient in *mdr1a* and / or *mdr1b* are increasingly employed to screen drugs as possible substrates of P-gp at the BBB level, the expression pattern of *mdr1a* and *mdr1b* in rodent brains has been investigated in few studies. These have often involved the whole brain and employed different techniques. No studies specifically examining the regional distribution of the two isoforms in the brain have been reported. In mice, one study using DNA-RNA hybridisation found both *mdr1a* and *mdr1b* in homogenised whole brain tissues, although the latter at lower level (Croop et al, 1989). In rats, only *mdr1a* is thought to be present in the cerebral microvessels *in vivo* (Barrand et al, 1995; Regina et al, 1998), although *mdr1b* has also been detected at very low level in whole brain tissues using RT-PCR, a more sensitive method than immunohistochemistry (Regina et al, 1998). In addition, brain levels of P-gp substrates can reach one to two orders of magnitude higher in *mdr1a* knockout or *mdr1a/1b* "double-knockout" mice compared to wild-type mice, whereas similar brain levels are found in *mdr1b* knockout mice and normal mice (Schinkel et al, 1997). This may suggest that *mdr1a* is the

predominant functioning P-gp in mouse BBB. However, expression of *mdr1b* confined to certain discrete at risk region(s) in the rodent brain conferring "extra protection" cannot be ruled out by results from these studies in which the whole brain was examined.

4.1.2 Cellular location of expression

The cellular location of P-gp in the brain has been the subject of much debate. If cerebral P-gp is expressed by cells other than the capillary endothelium, attempts to inhibit its action pharmacologically might have knock-on effects on neuronal and glial functions. The use of different methodologies has made comparison of findings across published studies problematic. With few exceptions (Pardridge et al, 1997), most authors report detection of P-gp by autoantibody staining or Western blotting on capillary endothelium and not on normal brain parenchymal cells (Cordon-Cardo et al, 1989; Seetharaman et al, 1998). Diseased astrocytes are the only exception to this observation (Bietzmann et al, 1994; Tishler et al, 1995). The highly conserved nature of the *mdr* genes between human and rodents suggests that they may have a similar distribution in rats. Western immunoblotting has demonstrated the presence of P-gp in isolated microvessels from rat brain cortex (Barrand et al, 1995; Beaulieu et al, 1997; Regina et al, 1998). However, using RT-PCR, Regina and co-workers (1998) found a very low level of *mdr1b* (but not *mdr1a*) expression in whole cerebral cortex homogenates but not in isolated cortical microvessels. This led the authors to conclude that the *mdr1b* isoform is expressed by brain parenchymal cells. In addition, *mdr1b* mRNA can be detected in primary culture of brain endothelial cells grown from capillary fragments isolated from rat cortical grey matter (Barrand et al, 1995; Regina et al, 1998). Whether it is also expressed in other cultured brain cells, such as astrocytes or neurones, has not been investigated.

4.2 Aims

The aims of this study were threefold. Firstly, the technique of quantitative RT-PCR developed for measuring mRNA concentrations was validated by testing the inter-assay variation. Secondly, regional expression of *mdr1a* and *mdr1b* genes, both qualitative and quantitative, in the rat brain was determined. Finally, RT-PCR was employed to determine the expression of *mdr1a* and *mdr1b* in primary cultures of rat cortical astrocytes and neurones. The rat was chosen instead of the mouse since its relatively larger brain allows dissection into regions of adequate size for reliable mRNA measurement.

4.3 Methods

4.3.1 Animals

Adults rats were obtained from Harlan Olac (Bicester, UK). Neonates and embryos for cell culture studies were obtained from a colony of Sprague-Dawley rats at the Central Research Facility at the University of Glasgow. All animals were housed in a controlled temperature/humidity environment with day/night cycle and access to food and water *ad libitum*. All experimental work was governed by the Animals (Scientific Procedures) Act, 1986 (UK).

4.3.2 Regional analysis

Four male Sprague-Dawley rats at 12 weeks of age were studied. They were sacrificed by cervical dislocation followed by decapitation. Each brain was quickly removed and dissected into seven regions, namely frontal cortex, dorsal cortex, cerebellum, pons, midbrain, hippocampus, and olfactory bulbs as previously described (Glowinski and Iversen, 1966). Each region was washed in ice cold saline, blotted dry, and immediately snap frozen in liquid nitrogen. Frozen tissues were stored at –70°C until required.

4.3.3 Primary culture of cerebral cortical astrocytes

Primary culture of cortical astrocytes was performed as previously described (Leach et al, 1996). The cerebral cortices of one day-old rat pups were removed under aseptic conditions and cleared of attached olfactory bulbs, basal ganglia, hippocampal formations and meningeal membranes. The dissected neopallia were then cut into small cubes (0.5 mm^3) by two passes (at 90^o) in a McIlwain tissue chopper (Mickle Laboratory Engineering Company Ltd, Gomshall, UK). The chopped tissue was transferred to a sterile glass filter (80 μm nylon mesh; Lockertex Ltd., Warrington, UK) and the filtrate collected in a sterile beaker. The chopped material was washed through the filter with culture medium (Dulbecco's modified Eagle medium (DMEM) supplemented with 20% (v/v) horse serum, 2.5 mM L-glutamine, 100 I.U./ml penicillin, and 100 μg/ml streptomycin, all obtained from Gibco BRL, Paisley, UK) to give a final volume of 3 ml per brain. The filtrate was then passed through a sterile needle (BD Microlance 21G 0.8 x 40) three times. The volume of the resulting suspension was adjusted with culture medium to allow a 3 ml aliquot per culture dish in a ratio of 1 brain to 3 dishes. A 3-ml volume of the final cell suspension was plated onto 60 x 15 mm Falcon Primaria culture dishes (A+J Beveridge, Edinburgh, UK). The cultures were maintained at 37^0C in an environment of 95% air / 5% CO_2 with a humidity of ≥ 90%. The culture medium (3 ml) was replaced every 3 - 4 days throughout. The horse serum concentration was reduced to 10% at the first medium change with a final reduction to 5% at the second change. After 14 days in culture the medium was supplemented with 0.25 mM 3′5′-dibutyryl cyclic adenosine monophosphate (cAMP; Sigma, Poole, UK) to induce cell differentiation. Expression of *mdr1* genes in the cultures was determined by RT-PCR 7 days after supplementation with cAMP.

4.3.4 Primary culture of cerebral cortical neurones

This method was devised from modifications of the methods of Larsson and co-workers (1981), Yu and co-workers (1984), and Courtney and colleagues (1990).

4.3.4.1 Reagents

Poly-D-lysine solution was prepared by dissolving 1.5 mg poly-D-lysine in 100 ml borate buffer (pH 8.4). Solution B consisted of 0.25% D-glucose, 0.3% bovine serum albumin (BSA), and 0.0382% $MgSO_4$ in phosphate buffered saline (PBS). Trypsin solution was prepared by dissolving 50mg of trypsin in 20ml of solution B. Concentrated DNase/soya bean trypsin inhibitor (SBTI) solution consisted of 2.8% DNase I, 5% SBTI, and 0.0382% $MgSO_4$ in Solution B. A weak DNase solution was prepared by diluting 3.2ml of the concentrated DNase/SBTI solution to 20ml with Solution B. The borate buffer was prepared by adjusting the pH of 15 μM boric acid to 8.4 with 1 M NaOH. The culture medium consisted of minimal essential medium (MEM) supplemented with 20% (v/v) horse serum, 2 mM L-glutamine, 7 μM para-aminobenzoic acid (PABA), 100 μI.U./litre insulin, 0.6% D-glucose, and 0.14% KCl. The culture medium was further supplemented with 40 μM cytosine arabinoside (ARA-C), where indicated, to remove all replicating, non-neuronal cells. All solutions were prepared under sterile conditions, using sterile water where required and filter sterilised through a 0.2 μm pore filter prior to use and/or storage. Solutions were stored sterile at 4^0C for up to 5 days. Liquid reagents were obtained from Gibco BRL (Paisley, UK) while solid ingredients were bought from Sigma (Poole, UK).

4.3.4.2 Tissue preparation

Tissue for cell isolation was removed under aseptic conditions. Pregnant female rats, at 15-17 days post-conception, were sacrificed by a blow to the head followed by cervical

dislocation. The abdomen was opened and all foetuses removed into a culture dish containing MEM. The membraneous-like skull surface was removed with forceps and the cerebral cortices resected with a delicate pinch between the points of a pair of curved watchmaker's forceps. The dissected cortices were transferred to a sterile universal tube containing 6 ml of MEM prior to cell isolation.

4.3.4.3 Cell isolation

Isolated cerebral cortices were chopped into small cubes (0.375 mm^3) by two passes (at 90^o) in a McIlwain tissue chopper. Chopped tissue was transferred to a sterile 50 ml Falcon tube containing 20 ml trypsin solution and gently agitated to reduce clumping. A sterile plastic pipette was used to aid this process. Tubes were capped, shaken gently, and incubated for 20 minutes at 37^0C. After incubation, 20 ml of weak DNAse/SBTI solution was added and tubes mixed gently. Cells were pelleted by gentle centrifugation (2000 rpm for 1 minute). The supernatant was discarded and 1 ml concentrated DNAse/SBTI solution added. Cells were resuspended by trituration of the cell pellet suspension through a sterile glass pipette. The cell suspension was transferred to a 15 ml Falcon tube, a further 1ml of concentrated DNAse/SBTI solution added and trituration repeated through a sterile glass pipette with a narrowed end. Underlying the cell suspension with 2ml of 4% BSA using a "kwill" filling tube facilitated decontamination of the cells. Cells were again pelleted by centrifugation at 2000 rpm for 5 minutes. The supernatant was discarded and the cells resuspended in 2ml culture medium. A ten-fold dilution of this cell suspension (90 µl culture medium + 10 µl cell suspension) was prepared and 10µl employed for cell counting in a haemocytometer. The total number of cells was calculated and the volume of the cell suspension adjusted with culture medium to give 1.25 million cells per ml. Falcon Primaria culture dishes (35 mm^2) had been pre-coated with 2 ml poly-D-lysine solution for 1 hour at room temperature and

allowed to air dry. A 2 ml volume of cell suspension was plated on each dish and plates were incubated at 37^0C for 30 minutes. Thereafter the medium was replaced with a further 2 ml of culture medium to remove all non-attached cells.

4.3.4.4 Culture maintenance

The cultures were maintained at 37^0C in an environment of 95% air / 5% CO_2 with a humidity of ≥ 90%. After 48 hours in culture the medium was replaced with 2 ml culture medium containing 40 μM ARA-C to eliminate all non-neuronal cells. At this point the horse serum concentration was reduced to 10%. The horse serum concentration remained at 10% thereafter. Twenty-four hours later (day 3) and again on day 6 the medium was again replaced with 2 ml culture medium without ARA-C. Cultures were seen to be fully mature and ready for use between day 8 and day 10 and were viable for up to 15 days.

4.3.5 Qualitative and quantitative determination of *mdr1* mRNA

Total RNA was extracted from brain tissues and cell cultures and cleaned as described in Part III; Sections 3.2 and 3.3. Qualitative RT-PCR was performed to determine whether *mdr1a* and *mdr1b* were expressed in the various brain regions. If so, concentrations of the mRNA were measured by quantitative RT-PCR as described in Part III; Section 3.5.

4.3.6 Inter-assay variation of quantitative RT-PCR

To measure inter-assay variation, one of the samples homogenised in RNA Denaturing Solution was split into four specimens, which were then processed individually on separate days for the remaining RNA extraction procedure and to the final analysis of quantitative RT-PCR results.

4.3.7 Statistical analysis

The inter-assay and inter-individual variability of *mdr1a* and *mdr1b* mRNA levels was assessed by calculating the standard error of the mean values. The mRNA concentrations in different regions of the brain were expressed as the mean values ± standard error, and compared by one-way analysis of variance with Dunnet's correction for multiple comparisons. Statistical calculation was performed using Minitab for Windows (version 11.21).

4.4 Results

4.4.1 Qualitative regional expression of *mdr1* genes

The same pattern of expression was observed in all four rats. Using primers specific for *mdr1a*, a fragment corresponding to the expected size (351 bp) was amplified from total RNA prepared from all seven brain regions dissected. However, use of primers specific for *mdr1b* resulted in the amplification of an expected 326-bp fragment to a significant level with RNA prepared from the hippocampus only (Figure 28). The level of expression of *mdr1b* in the other regions was below the quantifiable lower limit of the assay. Attempts to increase the RT-PCR products of *mdr1b* to fall within the quantifiable range were unsuccessful.

4.4.2 Inter-assay variation

Inter-assay variation of *mdr1a* mRNA level was assessed using a homogenised sample of cortex. Since *mdr1b* was only expressed at quantifiable level in the hippocampus, inter-assay variation of *mdr1b* mRNA level was assessed using homogenised hippocampi from one of the rats.

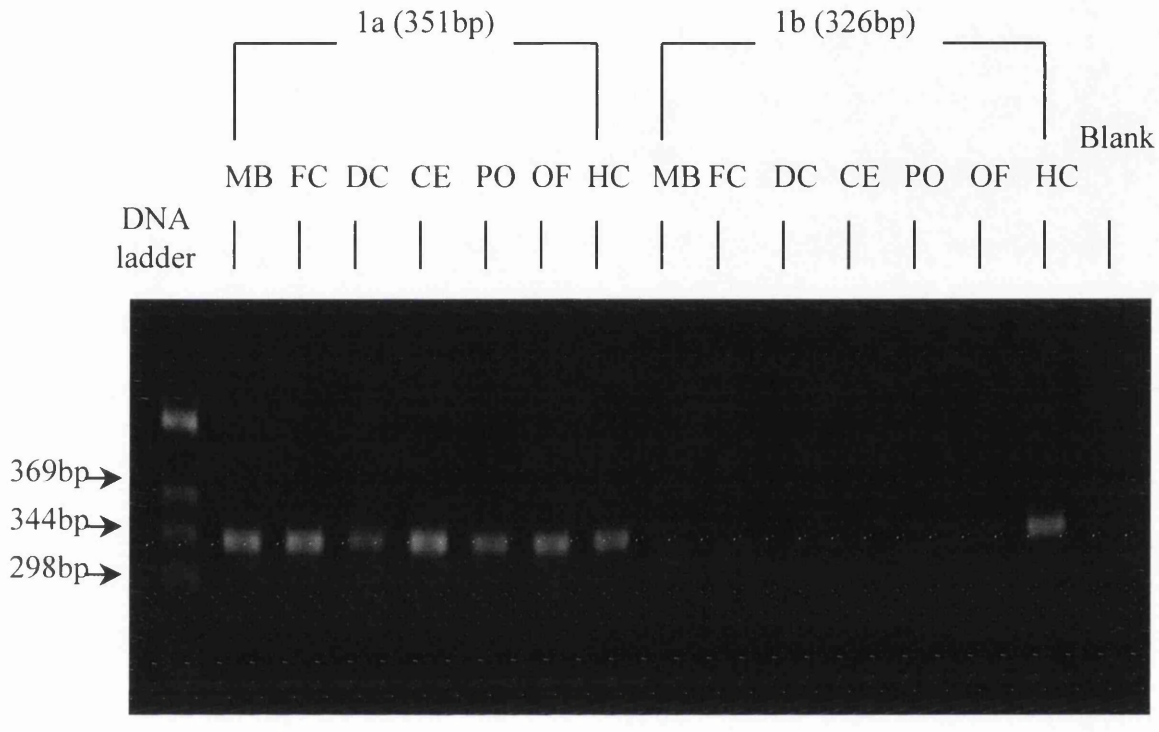


Figure 28. Regional expression of *mdr1a* and *mdr1b* in the rat brain.
MB=midbrain, FC=frontal cortex, DC=dorsal cortex, CE=cerebellum, PO=pons
OF=olfactory bulb, HC=hippocampus

The mean concentration of *mdr1a* mRNA measured in four separate analyses of the same cortical sample was 12320 molecules/ng total RNA, with a standard error of 301 molecules/ng total RNA (2.4%). The mean concentration of *mdr1b* measured in four separate analyses of the same hippocampal sample was 2223 molecules/ng total RNA, standard error 101 molecules/ng total RNA (4.6%).

4.4.3 Regional expression of *mdr1a* and *mdr1b* genes

Figure 29 shows the level of *mdr1a* expression as measured by concentration of mRNA. This did not differ significantly among the different brain regions. *Mdr1b* mRNA level was only high enough to be quantified in the hippocampus (Figure 30).

4.4.4 Expression of *mdr1a* and *mdr1b* genes in cortical astrocytes and neurones

Using primers specific for *mdr1a*, a fragment corresponding to the expected size (351 bp) was amplified from total RNA prepared from primary cultures of both cortical astrocytes and neurones (Figure 31a). Similarly, use of primers specific for *mdr1b* resulted in the amplification of an expected 325-bp fragment with RNA prepared from cultures of both cell types (Figure 31b).

4.5 Discussion

The narrow inter-assay variations (standard error <5% of mean values) in mRNA levels measured suggest the technique of quantitative RT-PCR developed for this purpose is reproducible for the determination of rat *mdr1a* and *mdr1b* mRNA. Marked inter-individual variation in *mdr1a* expression level was noted in some regions, particularly the cerebellum (standard error = 29% of mean values). Wide variation in expression level has also been noted in human studies (Part III; Section 1.2.7).

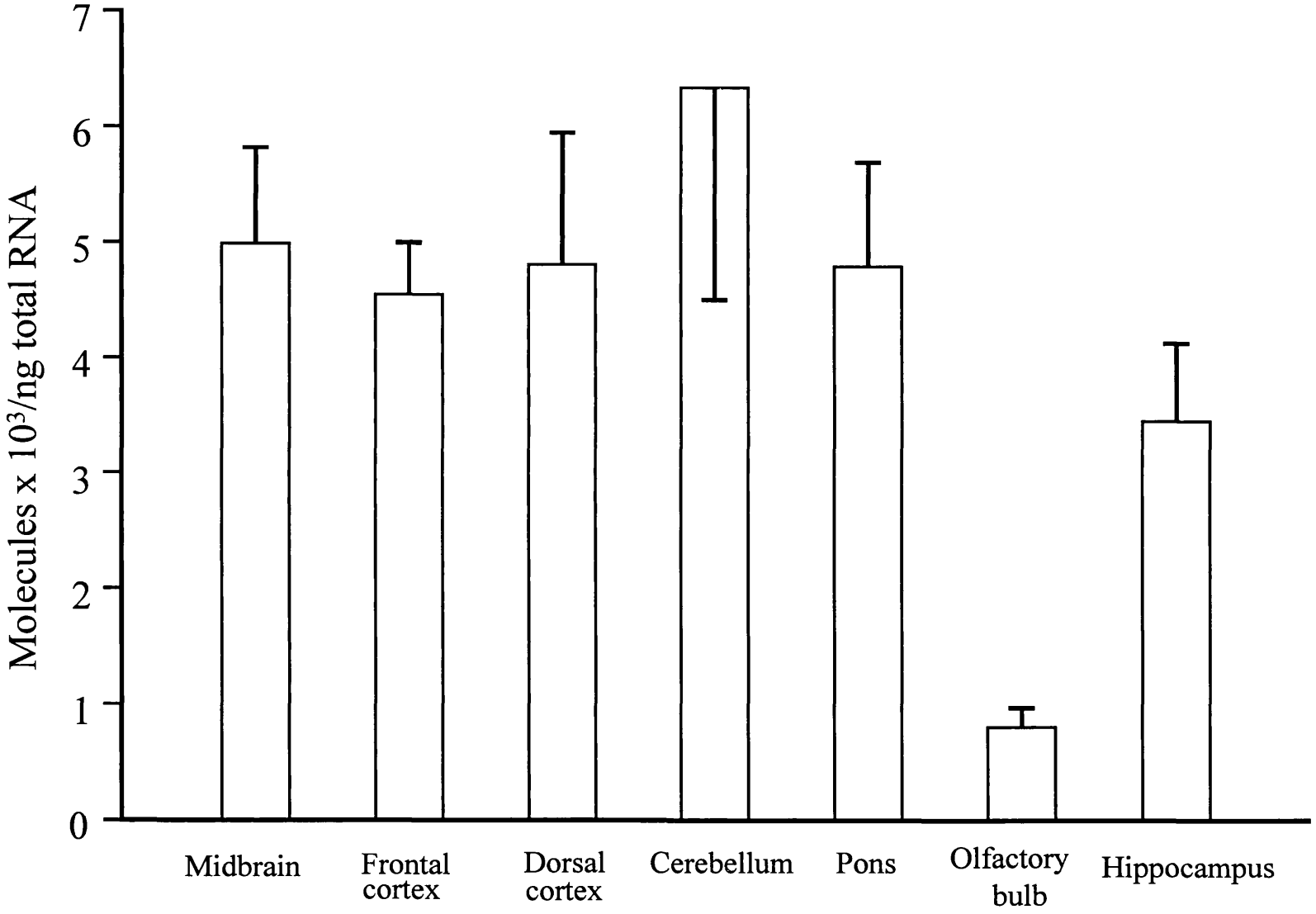


Figure 29. Concentration of *mdr1a* mRNA in various rat brain regions (n=4). Bars indicate standard error of the mean.

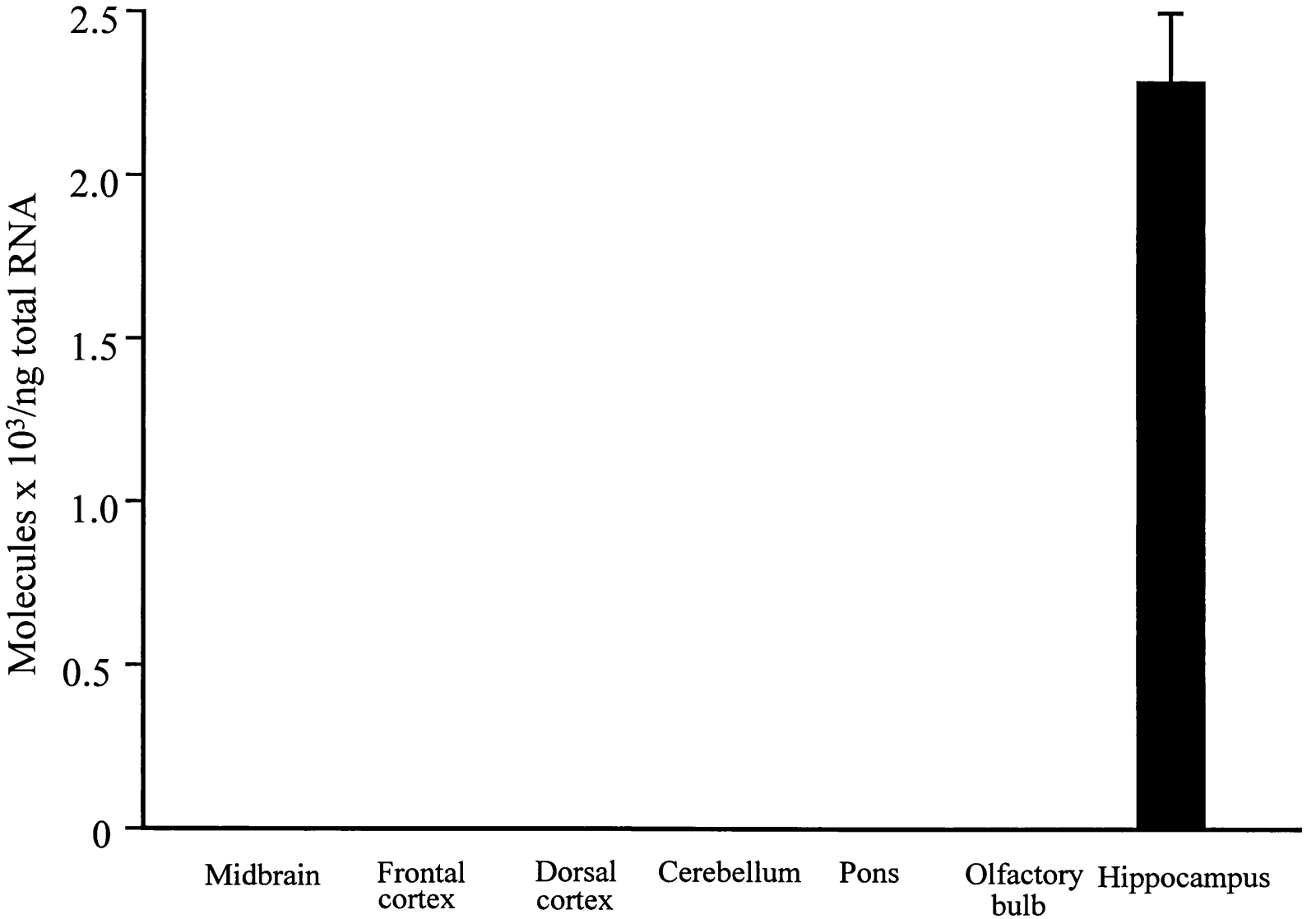


Figure 30. Concentration of *mdr1b* mRNA in various rat brain regions (n=4). Bar indicates standard error of the mean.

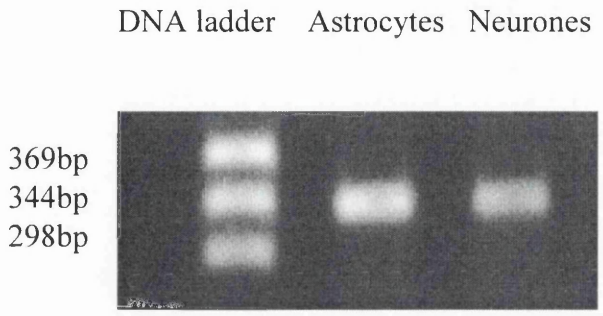


Figure 31a. Expression of *mdr1a* in primary cell cultures.
Expected band size = 351bp.

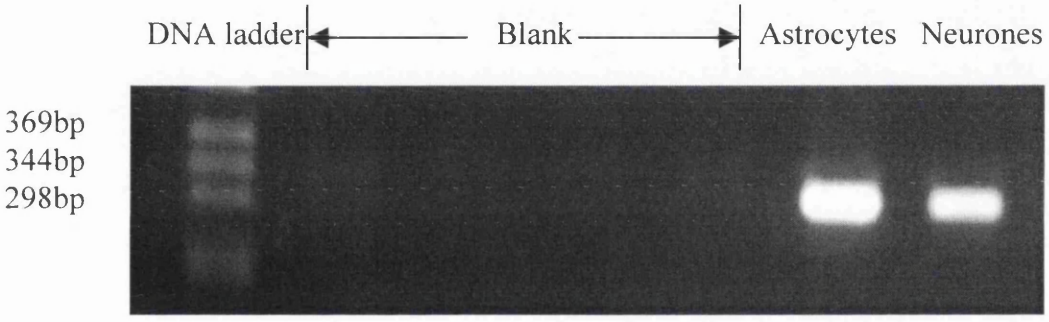


Figure 31b. Expression of *mdr1b* in primary cell cultures.
Expected band size = 326bp.

Although *mdr1* P-gp is increasingly recognised to play an important role in maintaining the mammalian BBB (Part III; Section 1.2.5), differential regional expression of the *mdr1* genes in the brain has not been reported. Results from the present study suggest that while *mdr1a* is expressed throughout the rat brain at comparable levels, *mdr1b* is only expressed to an appreciable amount by the hippocampus. Although the cellular location of *mdr1b* mRNA in the hippocampus could not be determined, a reasonable extrapolation of findings from the literature suggests that it is likely also to be present in capillary endothelium, serving similar functions as *mdr1a* P-gp (Part III; Section 1.2.5).

Why both *mdr1* isoforms should be expressed to a significant level in the hippocampus alone is unclear. The hippocampus is recognised to play a crucial role in higher cognitive functions, including memory, learning and spatial orientation, as well as emotion in mammals (Sutula et al, 1994; Parent, 1996). Virtually all aspects of memory processed by the hippocampal system can be referred to its function as a temporary memory store, believed to be effected via its exquisite anatomical and physiological plasticity (Lynch et al, 1990; Akhondzakeh, 1999). Both long-term potentiation and depression, which produce long-lasting changes in the efficacy of information processing at the synaptic site, are prominent in the hippocampus (Lynch et al, 1990; Bliss and Lomo, 1973; Akhondzakeh, 1999; Nayak and Browning, 1999). Lesions to the hippocampus produce memory deficits (Squire, 1992), as exemplified by the classic report of patient H.M. who developed profound anterograde amnesia with little change in other cognitive functions following bilateral medial temporal lobe resection (Scoville and Milner, 1957). HS is a common finding in elderly people with dementia (26% in one series; Dickson et al, 1994). HS/MTS is the most common pathological finding in refractory temporal lobe epilepsy (Part I; Section 1.4.2). The presence of HS is associated with generalised cognitive impairment in

intelligence, language, visuospatial functions and material-specific memory when compared to patients with temporal lobe epilepsy without HS (Hermann et al, 1997).

The presence of both *mdr1a* and *mdr1b* P-gp in the mammalian hippocampus might, therefore, have evolutionary significance by their enhanced ability to extrude xenobiotics from this particularly important but plastic brain structure. However, this "extra protection" might hinder pharmaceutical efficacy by limiting drug access when the hippocampus becomes diseased. This mechanism might conceivably contribute to the particularly pharmacoresistant nature of MTS-related epilepsy compared with other cerebral lesions (Part II; Section 4).

Such near-exclusive expression of *mdr1b* in the hippocampus might help to explain why *mdr1b* was not detected in microvessels isolated from the cortex (Barrand et al, 1995; Regina et al, 1998), but was present in "whole brain" (Croop et al, 1989). In addition, this distribution pattern of the *mdr1* P-gps might account for the similar level of digoxin, a strong P-gp substrate, measured in the brains of *mdr1b* (-/-) mice and *mdr1b* (+/+) mice (Schinkel et al, 1997), assuming the mouse displays the same expression pattern as the rat. Comparing the hippocampal levels of the drug in the two mouse genotypes might have yielded different results.

In cell culture, both *mdr1* isoforms were expressed at similar levels by astrocytes and neurones. However, P-gp has not been visualised in normal brain parenchyma in man (Cordon-Cardo et al, 1989; Tishler et al, 1995). It has been shown that whereas only *mdr1a* is expressed in vivo in rat brain microvessels, *mdr1b* is also expressed in cultured endothelial cells (Barrand et al, 1995). In view of the highly inducible nature of P-gp (Part

III; Section 1.2.7), it is likely that the expression of *mdr1b* in astrocytes and neurones is largely, if not entirely, induced by the culturing process itself.

In summary, while these data do not rule out expression of *mdr1* in normal brain parenchyma (proof would require analysis of brain tissues without the intricate microvessels which is not technically feasible), together with most literature reports, they suggest that P-gp is likely to be located primarily, if not exclusively, on the capillary endothelium of the BBB. It is possible that the additional expression of *mdr1b* in the hippocampus provides an enhanced protection of this sensitive and important brain region. This would have implications for those therapeutic agents, such as AEDs, which often require access to the hippocampus to exert their effects.

Further studies should include confirmation of *mdr1b* P-gp in the hippocampus by immunocytochemistry employing specific antibodies (Barrand and Twentyman, 1992). Determination of any differential regional expression of P-gp in man may only be practically and reliably investigated by non-invasive functional *in vivo* assays (Part III; Section 1.2.8). If the hippocampus were armed with extra functioning P-gp, AEDs that are substrates would be expected to reach lower level in this structure than other regions. Testing of this hypothesis requires measuring drug levels within specific brain regions by sensitive techniques, which have been attempted for a few drugs in very few studies (Rambeck et al, 1993; Cornford et al, 1996; Darius et al, 1999).

5 Effects of seizures in genetically epilepsy-prone rats

5.1 Introduction

5.1.1 Phenotype

Genetically epilepsy-prone rats (GEPRs) were developed originally from a colony of Sprague-Dawley rats in the late 1950s by selective breeding based on their susceptibility to sound-induced seizures (Laird and Jobe, 1987). These animals possess an inborn vulnerability to audiogenically induced seizures, which is usually displayed from around 2 to 4 weeks after birth and continues through adulthood (Thompson et al, 1991). Two distinct colonies have been developed based on the severity of the audiogenic seizures (AGS). The moderate seizure GEPRs (GEPR-3) exhibit a running, bouncing fit which terminates in generalised clonus with loss of righting reflex. The most commonly studied strain is GEPR-9 which displays the most severe form of AGS beginning with a very brief running phase, followed by a discrete clonic phase, terminating in tonic hind limb extension and post-ictal depression (Dailey et al, 1996; Faingold, 1999). Although the original GEPR breeding stock was selected for its sensitivity to acoustic stimulation, subsequent work suggests that these animals can exhibit spontaneous seizures and have increased susceptibility to a variety of other physical and chemical seizure stimuli (Laird and Jobe, 1987; Buchhalter, 1993).

All major established AEDs have been shown to be effective in preventing AGS in GEPRs (Reigel et al, 1986), although sensitivity varies with the strain (Dailey and Jobe, 1985). GEPR-9s are more sensitive than GEPR-3s to the anticonvulsant effect of PHT and CBZ. ESM is equally effective in both strains. VPA is more effective in GEPR-3s than GEPR-9s. It has been suggested such response patterns may be useful in identifying agents with different clinical indications, corresponding to tonic-clonic seizures, absence seizures, and

broad seizure spectrum, respectively (Laird and Jobe, 1987). Some compounds (e.g. chlorpromazine, haloperidol, baclofen) that possess anticonvulsant effects in other animal models (e.g. MES, scPTZ), but not in man, are devoid of activity in GEPRs (Dailey et al, 1996). Notwithstanding breeding difficulties (Consroe et al, 1979), GEPRs have, therefore, proven to be one of the most useful animal models of inherited epilepsy, with behavioural, electrographic and pharmacological features similar to primary GTCS (Coffey et al, 1996).

5.1.2 Neuronal networks

The pathway that underlies AGS in GEPR-9s principally involves neuronal networks in the brainstem (Faingold, 1999). It is generally believed that AGS are initiated by high intensity acoustic input to the cochlea, which is damaged in GEPR-9s. The output of the lower auditory nuclei is projected via glutamate receptor-mediated excitation to the inferior colliculus in the midbrain. This region has been shown to play a critical role in AGS initiation. Bilateral lesions of the inferior colliculus inhibit seizures whereas electrical stimulation produces seizures (Buchhalter, 1993). Recordings from this region demonstrate spike-wave discharges immediately prior to (Faingold, 1999), and during the initiation of AGS (Ludvig and Moshe, 1989). Cortical spikes are observed after discharges in the inferior colliculus, although cortical ablation does not eliminate seizures (Ribak et al, 1988).

The deep layers of the superior colliculus develop rapid tonic firing 1 to 2 seconds prior to wild running, suggesting a role in the generation of convulsive behaviour. Firing ceases in the post-ictal period with eventual recovery. Just prior to tonic hind limb extension and throughout this phase, discharges can be seen in the pontine reticular nucleus and

periaqueductal gray, implying a critical role of these regions in the manifestation of the tonic-clonic seizure (Faingold, 1999).

The genetic basis of AGS in the GEPR is unknown, but inheritance is likely to be polygenic and autosomal dominant (Buchhalter, 1993). Neurochemically, extensive data suggest that GEPRs have widespread deficiencies in noradrenergic and serotonergic functions in the CNS, both presynaptically and postsynaptically (Buchhalter, 1993; Dailey et al, 1996; Hosford et al, 1997). Recent observations of reduced effectiveness of GABAergic inhibition and an excess of glutamate availability in the inferior colliculus suggest altered GABA function may also play a role (Faingold, 1999).

5.1.3 Aims

This study aimed to examine whether the expression of *mdr1a* and *mdr1b* genes may be induced by a single seizure in GEPRs. The main advantage of using GEPRs for this purpose is the ability to generate seizures without administration of chemoconvulsive agents, which themselves might be substrates and/or inducers of P-gp. Since the neuronal network of the GEPR is relatively well-delineated, any potential effect of seizures on *mdr1* expression can be compared between the brain regions that are involved in seizure generation with those that are not. It can be hypothesised that if *mdr1* expression could indeed be induced by seizure, the effect would be most marked in the midbrain and cortex which are involved in the seizure pathway with relative sparing of other areas.

5.2 Methods

5.2.1 Materials

Twenty adult male rats (8-10 weeks of age) were taken from an established colony of GEPRs bred in-house at the Institute of Psychiatry, London, under the supervision of Professor Brian S Meldrum. None had been exposed to audiogenic stimulation prior to the experiment.

The rats were randomised into four groups. Five rats remained naïve and served as the control group while the remaining 15 were exposed individually to a single audiogenic stimulation (110-120 dB; 12-16 Hz; maximum 60 s or until seizures began) as previously described (Smith et al, 1993). Five rats were sacrificed at 4 hours, five at 24 hours, and the remaining five at 7 days post-stimulation. All rats were kept in a controlled temperature and humidity environment with day/night cycle conditions and access to food and water *ad libitum* until sacrificed by decapitation. Their brains were removed and quickly dissected into four regions, namely midbrain, cortex, pons/medulla, and hippocampus (Glowinski and Iversen, 1966). The brain regions were placed individually in microcentrifuge tubes and immediately snap frozen in liquid nitrogen before being transferred to storage at $-70^{\circ}C$ until required.

5.2.2 Measurement of *mdr1* mRNA

Total RNA was extracted from brain tissues and cleaned as described in Part III; Sections 3.2 and 3.3. *Mdr1a* and *mdr1b* mRNA concentrations were measured by quantitative RT-PCR (Part III; Section 3.5).

5.2.3 Statistical analysis

Group results were expressed as the mean of absolute values ± standard error of the mean. Groups sacrificed at different time points after stimulation were compared to the control animals using one-way analysis of variance with Dunnet's correction for multiple comparisons. Statistical calculation was performed using Minitab for Windows (version 11.21).

5.3 Results

All but one of the stimulated GEPRs displayed motor seizures of either moderate (GEPR-3) or severe (GEPR-9) form in response to acoustic stimulation. *Mdr1a* mRNA was present in all regions of all stimulated and control rats, whereas *mdr1b* mRNA was detected at quantifiable levels in the hippocampus only.

Compared with controls, *mdr1a* mRNA concentration in the cerebral cortex rose at 4 hours after a single audiogenic stimulation (Figure 32). There was a further rise at 24 hours ($p<0.05$), after which the concentration declined, although it remained elevated at 7 days post-stimulation. A similar time-dependent response in *mdr1a* mRNA level was observed in the midbrain (Figure 33), although the increase did not reach statistical significance. Audiogenic stimulation did not significantly alter the mRNA levels of *mdr1a* in the pons/medulla (Figure 34) or hippocampus (Figure 35), or of *mdr1b* in the latter (Figure 36) at any of the time points investigated.

5.4 Discussion

Results from this pilot study suggest that seizures may induce changes in P-gp expression. A rise in *mdr1a* expression was observed at 4 hours after a seizure in brain regions that are

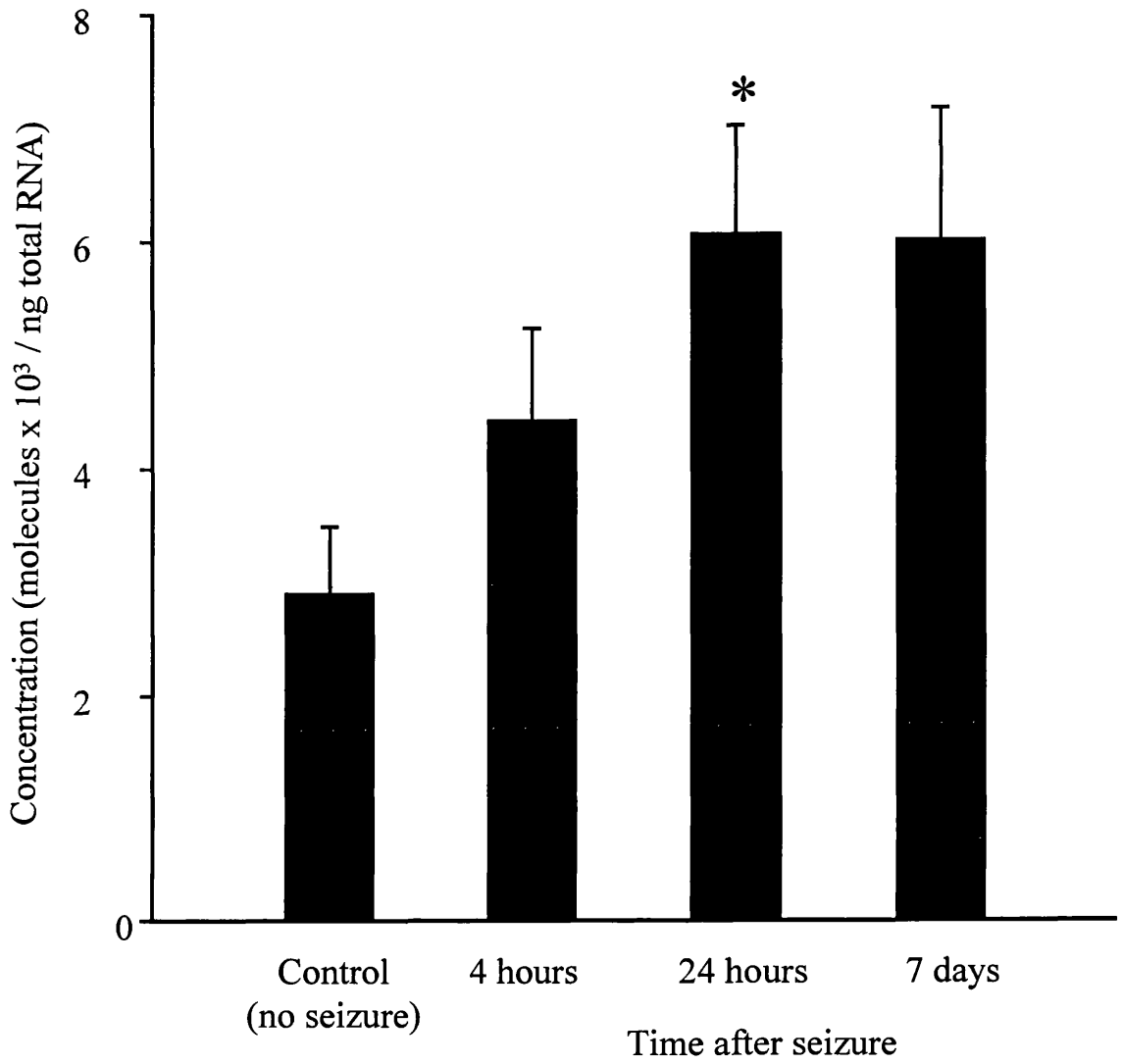


Figure 32. Concentration of *mdr1a* mRNA in cerebral cortex of GEPRs at various time points after a single audiogenic seizure. *$p<0.05$. Bars indicate standard error of mean.

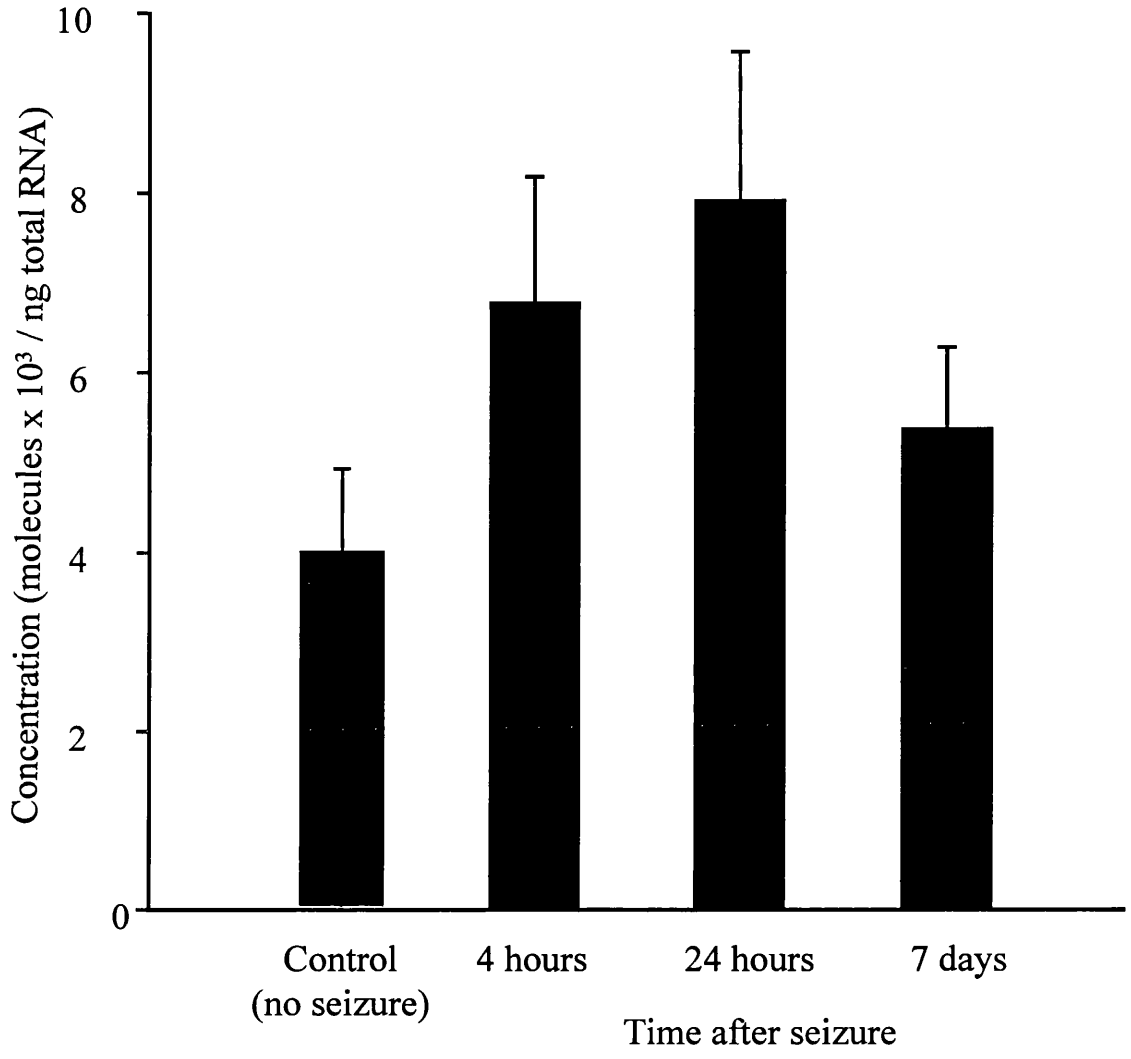


Figure 33. Concentration of *mdr1a* mRNA in midbrain of GEPRs at various time points after a single audiogenic seizure. Bars indicate standard error of mean.

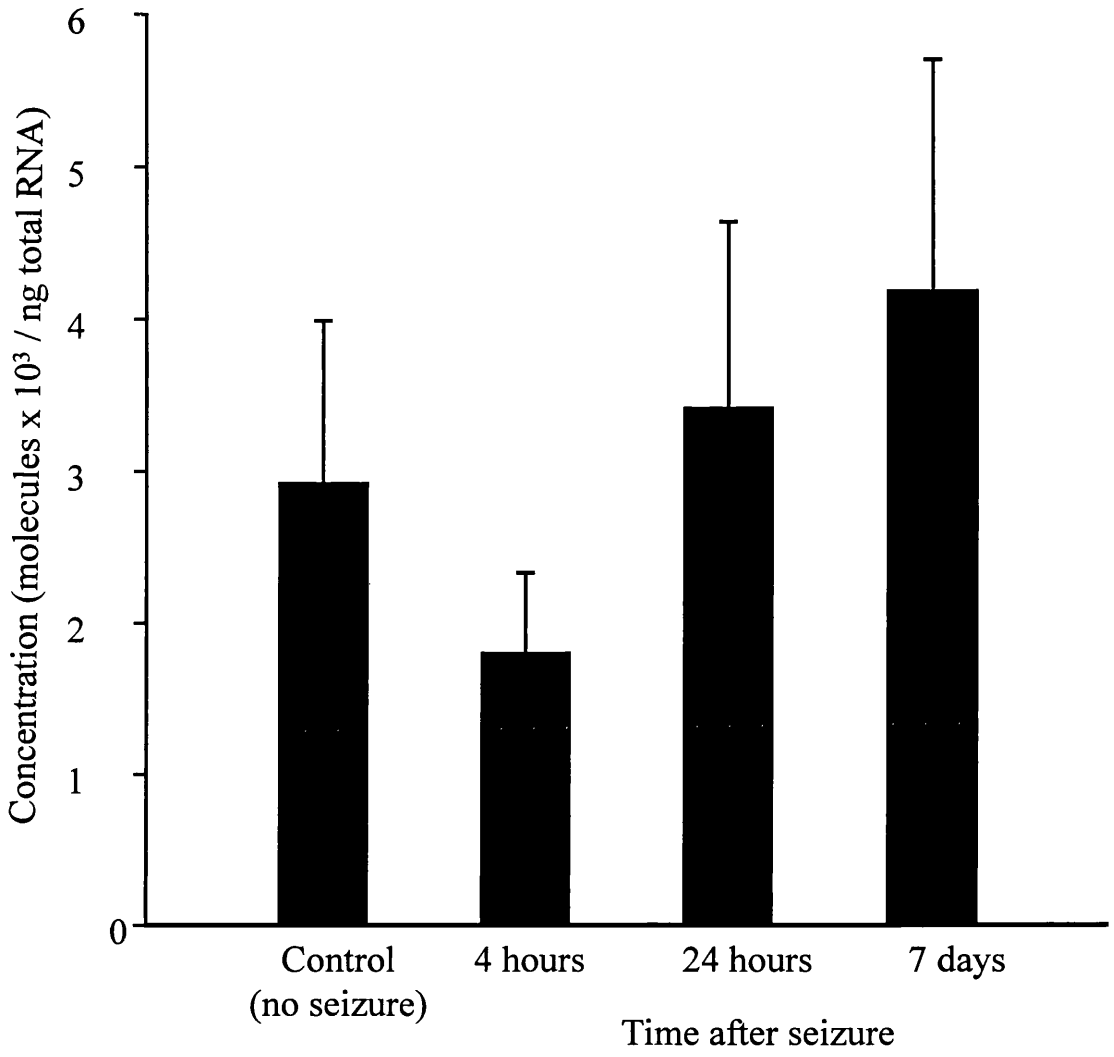


Figure 34. Concentration of *mdr1a* mRNA in pons/medulla of GEPRs at various time points after a single audiogenic seizure. Bars indicate standard error of mean.

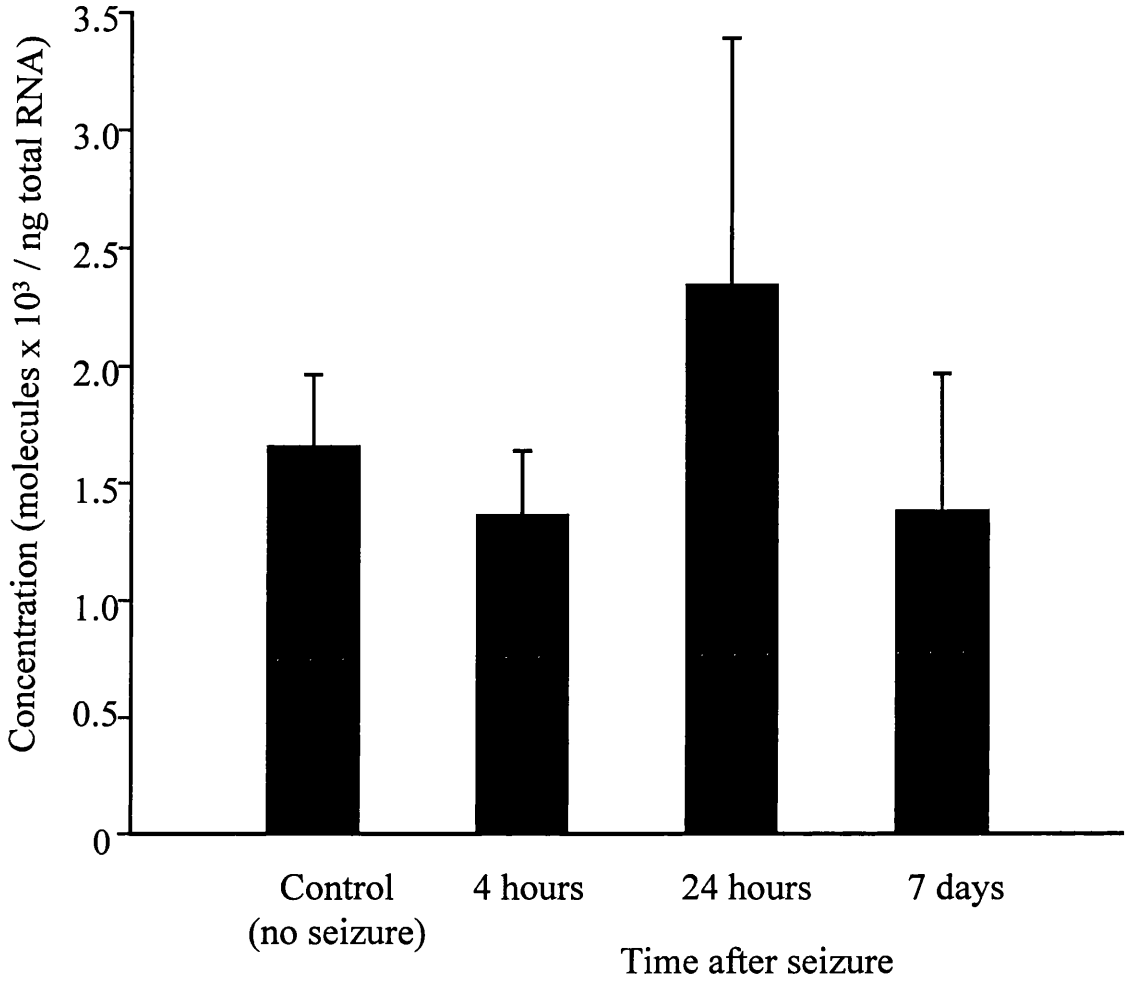


Figure 35. Concentration of *mdr1a* mRNA in hippocampus of GEPRs at various time points after a single audiogenic seizure. Bars indicate standard error of mean.

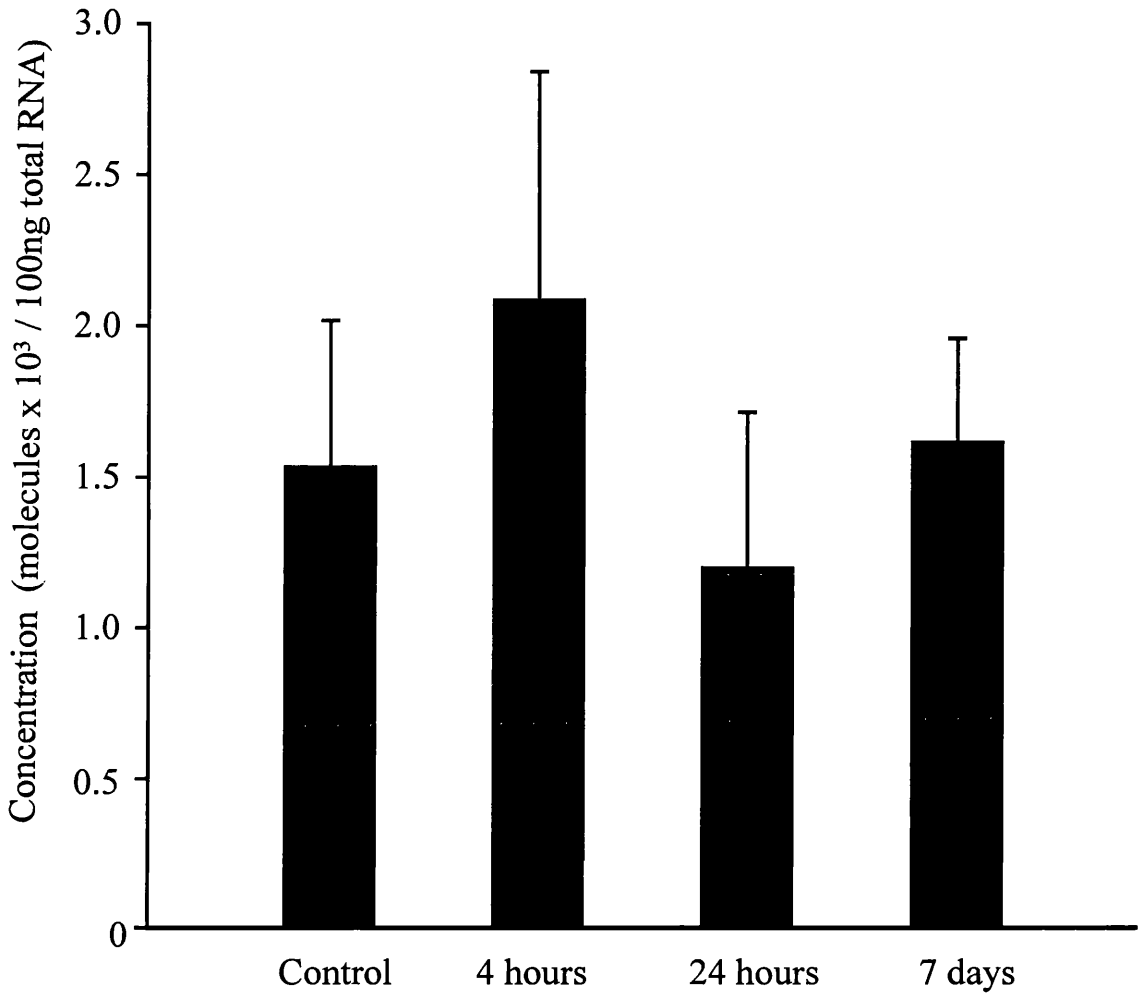


Figure 36. Concentration of *mdr1b* mRNA in hippocampus of GEPRs sacrificed at various time points after a single audiogenic seizure. Bars indicate standard error of mean.

known to be involved in the seizure propagating pathway. The maximum increase in expression in this study was observed at 24 hours. The temporal pattern suggests that the peak expression may be observed at some point between 4 hours and 7 days post-stimulation. Significant changes were observed in the cortex, a structure to which seizure activity originating in the midbrain (inferior colliculus) projects. Since chemoconvulsants (which might be P-gp substrates or inducers) were not used, these changes most likely represent response to the sound-induced neuronal discharges.

Adaptive changes of the BBB to various insults, including seizures, have long been recognised (Cornford and Oldendorf, 1986). Both animal and human data have traditionally suggested a non-specific transient increase in permeability of the BBB ictally (Cornford and Oldenforf, 1986; Duncan and Todd, 1991). More recently, activities of certain components of the BBB, for instance the glucose transporter Glut1, have been shown to be specifically up-regulated acutely during seizures (Cornford, 1999). Such transient up-regulation of glucose transport has been suggested to account partly for the ictal hypermetabolic state of a seizure focus detected by positron emission tomography using [^{18}F]-fluorodeoxyglucose (Cornford, 1999).

On the other hand, relatively little is known about the long-term changes at the BBB in epilepsy. Down-regulation of the Glut1 glucose transporter activity has been observed in the epileptogenic focus of patients with chronic partial epilepsy (Cornford, 1999). Thickening of the cerebral capillary basement membrane in tissues resected from patients with chronic epilepsy has been described (Kasantikul et al, 1983). Using concomitant intraoperative electrocorticographical recordings, Liwnicz and colleagues (1990) also found thickening of basement membrane in microvessels from spiking cortical regions

when compared to non-spiking control areas resected from patients with refractory partial seizures. These findings suggest a chronic reinforcement of the BBB in response to repeated seizures.

Since P-gp plays an important role in maintaining the integrity of the BBB (Part III; Section 1.2.5), the changes in P-gp expression observed in this study might reflect a prolonged post-ictal compensatory mechanism aimed at reinforcing the BBB, in an analogous manner to the structural alterations of the basal lamina described above. Whereas expression of the immediate early genes returns to basal level within 2 hours post-seizure (Burazin and Gundlach, 1996), *mdr1a* P-gp remained significantly over-expressed at 24 hours. It is possible that with repeated seizures, P-gp expression might become further increased, eventually progressing to a state of chronic elevation. A sufficient seizure frequency may, therefore, be required for the maintenance of over-expression, and for P-gp to exert a "drug-resistance" role in chronic epilepsy. This might partly explain the relationship between refractory epilepsy and number of pre-treatment seizures (Part II; Section 1), and the observation of increased P-gp expression in brain tissues surgically removed from patients with pharmacoresistant epilepsy (Part III; Section 1.3). Since some AEDs are substrates of P-gp (Part III; Section 2), over-expression of P-gp as a result of repeated seizures has the potential to inhibit their penetration of the BBB and limit their efficacy.

In conclusion, these preliminary data suggest that P-gp may be induced by a single seizure in brain regions of GEPRs involved in the seizure propagating pathway. A larger number of animals are required to confirm these findings. Further studies should include investigation of more post-seizure time points up to and beyond 7 days to better

characterise the temporal changes. The effect of recurrent seizures and prolonged seizures (status epilepticus) on P-gp expression should also be investigated. Finally, other seizure models, including those of focal and generalised origin, should be studied to determine whether these changes are confined to GEPRs or are a consequence of seizures in general.

6 Effects of laser-induced damage

6.1 Introduction and aim

Focal application of laser radiation was initially developed as a novel animal model of partial epilepsy by Dr Graeme J Sills, one of my PhD supervisors, at the Epilepsy Unit (Sills, 1994). The model was designed to impinge a laser beam of sufficient intensity upon a particular region of the cerebral cortex (in this case the primary motor cortex) to induce tissue damage which might become a local epileptic focus. The major advantage of such a procedure is the ability to employ a precise and quantifiably variable necrotic stimulus which could be reproduced with a high degree of accuracy in large groups of animals. In addition, the highly focused application of the laser beams permits the use of small animals, minimising expense and husbandry requirements.

The procedure was performed in 126 rats (78 laser-treated and 48 sham-operated) which were monitored for up to one year post-operatively. Less than 5% of the animals showed deviation from normal health and unexpected death occurred in less than 1%. In the laser-treated animals examined nine months post-operatively, a well-circumscribed lesion was observed which was approximately 1mm in diameter at the cortical surface, tapering to around 60 μm in cortical layer six, sparing the underlying white matter. Immunohistochemical staining revealed selective loss of neuronal cells and prominent gliosis and fibrosis without infiltration of chronic inflammatory cells. Consistent with histological findings, a reduction in local cerebral glucose utilisation and an increase in ligand binding to the peripheral-type benzodiazepine receptor (marker for brain damage) were confined to the lesioned area or its immediate vicinity (Sills, 1994).

Although overt seizure activity was not observed, these preliminary results suggested the procedure to be safe and effective in creating a reproducible focal cortical lesion with associated histological and biochemical changes. It might, therefore, serve as a useful model of P-gp induction, in much the same way as other mechanisms of physical injury, such as heat shock treatment in certain cell types (Part III, Section 1.2.7; Chin et al, 1990).

The aims of the following pilot study were to explore the hypothesis that *mdr1* gene expression may be induced by physical damage, in this case by focal laser application to the rat brain, and whether a temporal effect could be observed.

6.2 Methods

6.2.1 Study design

Nine adult male Sprague-Dawley rats (200-300g) were randomly assigned into three groups, corresponding to a sacrifice time of 1 week, 4 weeks, and 8 weeks post-procedure (n=3 each group).

6.2.2 Instrument

The lesioning radiation was delivered by a Synrad 10-watt CO_2 laser. It consisted of an extensive beam delivery and alignment apparatus, incorporating a zoom beam expander, a beam attenuator, a mirror block, a fine focus unit, and a magnifying lens. The invisible nature of CO_2 laser radiation necessitated the inclusion of a helium/neon (HeNe) laser which has no ionising action of its own but whose red beam could be employed for aiming purposes. The HeNe laser was incorporated into the beam delivery assembly, and adjusted to be in direct alignment with the main CO_2 beam. The desired laser output could be obtained by adjusting various parameters in the beam delivery apparatus. Absolute outputs

ranged from 0.2 to 10.0 watts at intervals of approximately 0.1 watt. A foot pedal was incorporated to initiate the CO_2 beam, allowing the operator to conduct the lesioning procedure in a safe, "hands-free" manner. An intrinsic timing device controlled the CO_2 laser output and allowed timed exposures of between 1 and 60 seconds at 1 second intervals.

6.2.3 Preparation of animals

Each operated animal was anaesthetised by placing it in a sealed chamber which was then flooded with 5% halothane delivered with oxygen (1.5 L/min). The animal was allowed to remain in the chamber for up to 5 minutes to ensure anaesthesia had progressed to a sufficiently deep stage.

6.2.4 Surgical procedure

On removal from the chamber, the animal was maintained under 1.5% halothane anaesthesia delivered via a face mask. The scalp was shaved and cleaned with a disposable alcohol swab and a midline incision of approximately 1.5cm in length was made with a scalpel. The skin was reflected in either direction and lightly clamped. The periosteal membranes overlying the right frontal and parietal bones of the skull were cleared with sharp forceps. A hand-held electric drill was used to produce a small burr hole of approximately 2mm diameter in the skull, over the right primary motor region of the cortex, approximately 3mm anterior and 3mm lateral to zero Bregma (Paxinos and Watson, 1982). The drilling time was kept to a minimum ($\leq$ 20 seconds) to prevent inadvertent heating of the skull. Care was also taken not to rupture the underlying dura mater.

6.2.5 Production of a cortical laser lesion

The animal was briefly removed from the anaesthetic apparatus and placed in the path of the red HeNe aiming laser beam. The position of the animal was adjusted until the HeNe beam impinged directly on the centre of the circle of exposed dura mater. At this point the CO_2 laser beam was initiated to deliver 0.5 watt in a 2-second period.

6.2.6 Recovery of animals

Following the lesioning procedure, animals were re-fitted with the anaesthetic face mask and the scalp incision was closed with 4-5 absorbable stitches. Animals were then placed in individual cages and monitored until consciousness was regained, and monitored thereafter on a twice daily basis. They were kept in a controlled temperature and humidity environment with day/night cycle conditions and access to food and water *ad libitum* until sacrificed by stunning followed by cervical dislocation.

6.2.7 Areas of interest and measurement of *mdr1* mRNA

Upon sacrifice, the brain was quickly removed and the cortex dissected free. The lesioned area was removed together with the adjacent margin in a cube of tissue of approximately 60-80mg in weight. A similar sized brain tissue was removed from the same area of the contralateral hemisphere to serve as control. Individual samples were placed in microcentrifuge tubes and immediately snap frozen in liquid nitrogen before being transferred to storage at –70°C until required. Total RNA was extracted from the brain tissues and cleaned as described in Part III; Sections 3.2 and 3.3. *Mdr1a* and *mdr1b* mRNA concentrations were measured by quantitative RT-PCR (Part III; Section 3.5).

6.2.8 Statistical analysis

Group results were expressed as the mean of absolute values ± standard error of the mean. *Mdr1* mRNA concentrations in the laser-damaged and contralateral brain tissues were compared by paired Student's t-test. Statistical calculation was performed using Minitab for Windows (version 11.21).

6.3 Results

Mdr1a mRNA was detected in all samples. Its concentrations at various time points post-injury are shown in Figure 37. There was no significant difference in the level of expression between the section circumscribing the damaged cortex and the contralateral region at any of the time points investigated.

Mdr1b mRNA was detected at an appreciable level only in the laser-damaged cortex 1 week post-injury (Figure 38). It was not present at a quantifiable amount in any sample at 4 weeks and 8 weeks.

6.4 Discussion

The limited literature available on pathological cerebral P-gp expression has focused on "natural" diseases, such as tumours (Matsumoto et al, 1991) and cortical dysplasia (Sisodiya et al, 1999). Whether external physical insult can induce P-gp expression *in vivo* has not been addressed. Focal laser application represents a novel technique in producing a localised injury to the cerebral cortex. Regulatory restrictions and limitation of facilities meant that only a small number of animals could be employed. This pilot study was, therefore, necessarily exploratory in nature. It was intended to examine the feasibility of

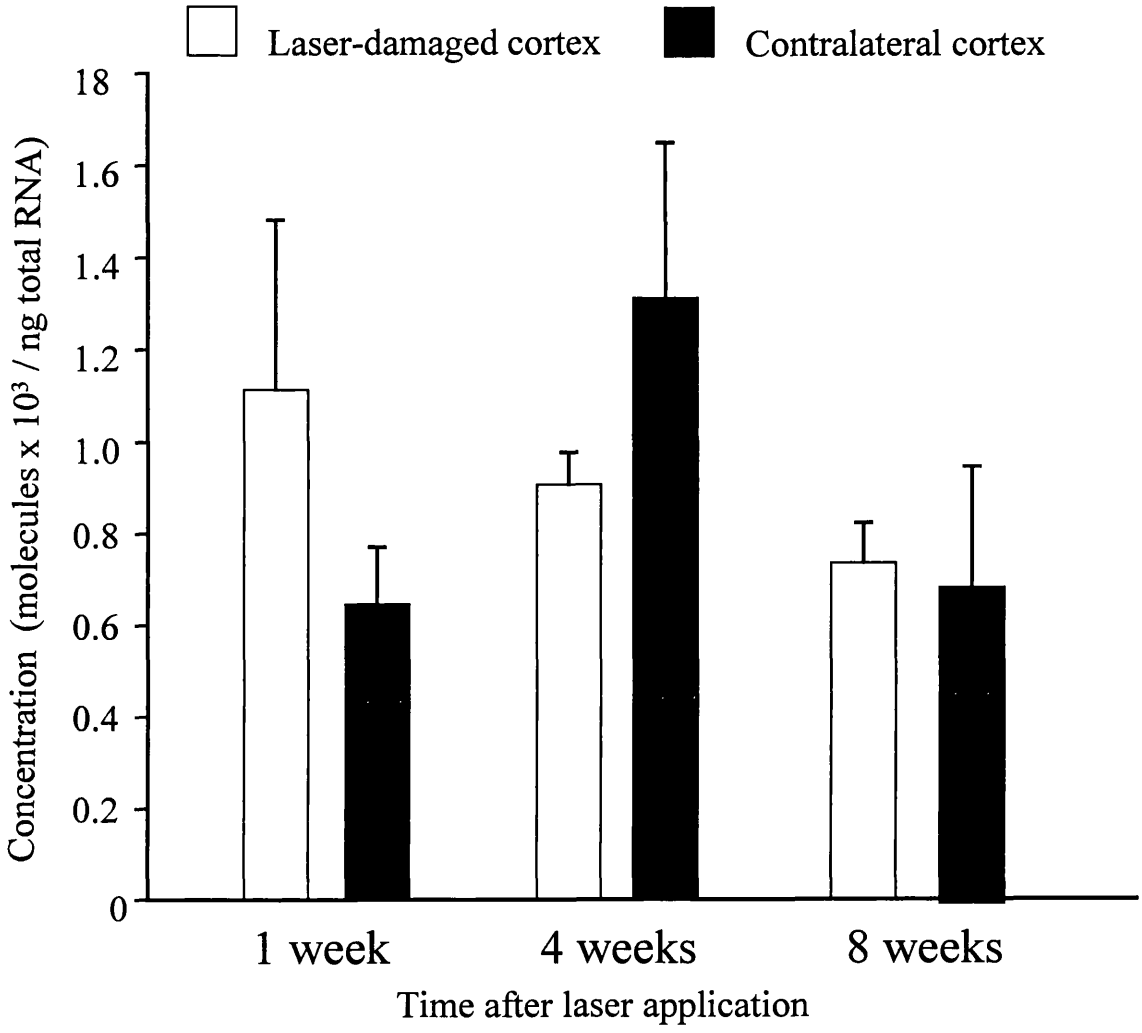


Figure 37. Concentration of *mdr1a* mRNA in laser-damaged cerebral cortex and contralateral cortex. Bars indicate standard error of mean.

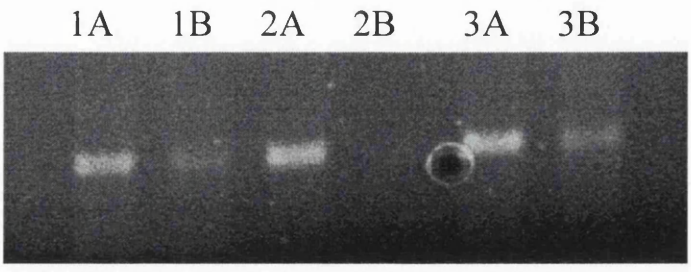


Figure 38. Expression of *mdr1b* in cerebral cortex of three rats 1 week after laser treatment. A=laser-damaged cortex; B=contralateral cortex.

this technique for the study of P-gp expression, and to generate results that might guide the design of more definitive experiments in the future.

These preliminary data suggest that the laser lesion may be associated with a transient expression of *mdr1b* in the damaged cortical region, detectable at 1 week post-injury. *Mdr1b* was not expressed to an appreciable level in the contralateral undamaged cortex, or in the damaged area at 4 and 8 weeks after injury. The relevance of this transient induction is unclear. If it is translated to an increased P-gp expression at the protein level, it might represent a component of the cellular changes in response to injury. However, its disappearance by 4 weeks post-injury casts doubts over its possible long-term implication. Concomitant electrophysiological study would be required to examine whether these changes were due to physical damage per se, or any resultant neuronal discharges. Extension of the observation period beyond 8 weeks is needed to investigate any potential long-term effects of damage on P-gp expression.

There was no statistical difference in the expression level of *mdr1a* between the damaged and undamaged cortical regions at any of the time points investigated. However, only three animals were studied in each group and whether the contralateral undamaged cortical area could be used legitimately as a control is open to question. It is possible that focal damage in one hemisphere could induce alterations in P-gp expression in contralateral areas. The two cerebral hemispheres are intimately interconnected by neuronal networks, as exemplified by the phenomenon of secondary epileptogenesis in animal models of focal epilepsy. In this scenario, a primary epileptogenic lesion is able to induce epileptiform behaviour in initially normal cell populations in the contralateral hemisphere via recurrent

discharges, creating a "mirror focus" which, in time, becomes an independent source of epileptic activity (Morrell, 1991).

Only limited conclusions can be drawn from these preliminary data. Clearly, a larger number of animals are required to explore the full potential of this technique to induce P-gp expression. Future studies should, perhaps, focus on 1 week post-injury in view of the changes in *mdr1b* expression observed. Sham-operated (animals undergoing the operative procedures without exposure to laser radiation) and naïve animals should also be used to control for the possible effects of the operation itself and anaesthetic agents on the expression of *mdr1* genes.

7 Expression of *MDR1* gene in human epileptogenic focus

7.1 Introduction and aim

Investigation of P-gp in animal models has clinical relevance only if its expression can be detected in human epilepsy. Although cerebral *MDR1* expression is well documented in the normal, and in some cases diseased, human brain (Part III; Sections 1.2.5 and 1.2.6), its presence in the brains of patients with epilepsy has been reported in only a few published case series (D'Giano et al; 1997; Lazarowski et al, 1999). Only one study attempted to measure its expression by a semi-quantitative technique (Tishler et al, 1995).

The present study aimed to test the sensitivity of the RT-PCR technique as described in Section 3 (Part III) for the detection of *MDR1* gene expression in brain tissues resected from patients with medically intractable epilepsy. If the assay technique is able to detect *MDR1* mRNA in such surgical specimens, measurement of gene expression using competitive internal standards, similar to the quantitative RT-PCR technique used for rat *mdr1*, may be developed for absolute quantification.

7.2 Methods

7.2.1 Patients

Brain tissues resected from two patients during epilepsy surgery were obtained after consent had been obtained to use the materials for research purposes.

Patient 1 was 41 years of age when the operation was carried out. He had had uncontrolled epilepsy since the age of 6 months following an attack of meningitis. The seizures were predominantly complex partial in nature, or simple partial consisting of aura only, with occasional secondary generalisation. He had been treated unsuccessfully with a

number of AEDs including PHT, CBZ and VGB. Neuroimaging and neurophysiological investigations all converged on his left mesial temporal lobe as the seizure focus and a left temporal lobectomy was performed.

Patient 2 was 11 years old when she underwent epilepsy surgery. She suffered febrile convulsion in her first year associated with herpes simplex virus infection. Since then she had been experiencing frequent complex partial seizures. A typical attack was heralded by an unusual smell, followed by aural automatism and staring without speech disturbance or loss of consciousness. Despite AED treatment, including PHT, LTG and TPM, she was having up to 16 seizures per month. Brain MRI showed right mesial temporal sclerosis. Inter-ictal EEG and SPECT also demonstrated right temporal abnormalities. A right anterior temporal lobectomy was performed.

7.2.2 Detection of *MDR1* mRNA

A small portion of the resected brain tissue from each of these patients was obtained fresh during the surgical procedure. The sample was placed in a sterile container and snap frozen in liquid nitrogen before being transferred to storage at –70°C until required. Total RNA was extracted from brain tissues and cleaned as described in Part III; Sections 3.2 and 3.3. RT-PCR (Part III; Section 3.5) was performed on the extracted RNA to determine the presence of *MDR1* mRNA.

7.3 Results

Using primers specific for *MDR1*, a strong band corresponding to the expected size (161 bp) was amplified from the total RNA prepared from brain specimens acquired from both patients (Figure 39).

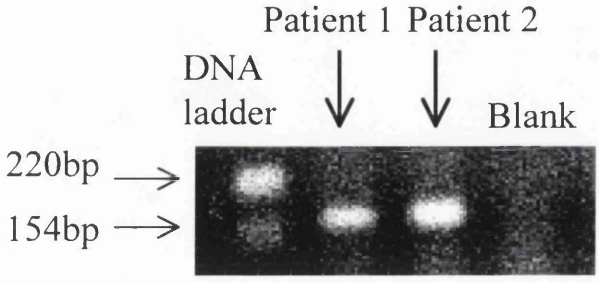


Figure 39. Expression of *MDR1* in brain tissues resected from patients with refractory temporal lobe epilepsy. Expected band size = 161.

7.4 Discussion

A significant amount of *MDR1* mRNA was detected in the brain tissues resected from these two patients with refractory temporal lobe epilepsy. The assay technique is, therefore, sufficiently robust for clinical specimens which are prone to handling degradation during resection.

Quantitative measurement was not carried out due to lack of control tissues. A previous semi-quantitative analysis found increased expression of *MDR1* in brain tissues resected from patients with refractory epilepsy compared to "normal" tissues (Tishler et al, 1995). A study to compare *MDR1* expression between epileptogenic brain tissues from patients with refractory epilepsy and "normal" tissues resected during removal of arteriovenous malformation is underway. Absolute quantification of *MDR1* mRNA will be made with use of competitive internal standards.

8 General discussion and conclusion

8.1 Discussion

The aim of this series of pilot experiments was to explore whether the drug transporter P-gp could play a role in the genesis of refractory epilepsy. It was hypothesised that over-expression of P-gp at the BBB might contribute to drug resistance by limiting AED access to the seizure focus.

Using *mdr1a* (-/-) mice that are devoid of cerebral P-gp, it was suggested that TPM, PHT and possibly CBZ may be substrates of P-gp at the BBB (Part III; Section 2). A reproducible, robust assay for quantifying *mdr* mRNA was developed, tested, refined and validated (Part III; Section 3), allowing the measurement of P-gp expression in various models in the subsequent pilot studies. P-gp is expressed in a region-specific manner in the rat brain. *Mdr1b* was found to be constitutively expressed to a significant level by the hippocampus alone, while *mdr1a* was expressed uniformly throughout the brain (Part III; Section 4). A single seizure appeared to result in a sustained increase in P-gp expression in the GEPR along the seizure generating pathway, without affecting other brain regions (Part III; Section 5). Physical trauma produced by focal application of laser is associated with a transient expression of *mdr1b* to an appreciable amount at 1 week, but did not affect P-gp expression when examined at 4 and 8 weeks post-injury (Part III; Section 6). Lastly, P-gp expression was detected in brain tissues resected from patients undergoing temporal lobectomy for drug-resistant epilepsy (Part III; Section 7).

Very few studies have examined the potential relationship between P-gp and epilepsy (Part III; Section 1.3). Little guidance in experimental design and methodology could, therefore, be gleaned from the literature. Regulatory requirements have placed further restrictions in

study techniques and protocols. These pilot studies were, therefore, necessarily exploratory in nature. They were primarily intended to examine the feasibility of the assay technique for the study of P-gp expression, and to generate results that might guide the design of more definitive experiments in the future. It is in this context that these results should be interpreted.

While only limited conclusions can be drawn from the experiments individually, when pieced together, these findings do strengthen the argument for a potential role of P-gp in the pathogenesis of refractory epilepsy, especially that related to MTS. The exclusive expression of *mdr1b* in the hippocampus might correlate with the particularly drug resistant nature of temporal lobe epilepsy (Semah et al, 1998). The low remission rate among patients with MTS (Part II; Section 4), and the relative insensitivity in a subset of amygdala-kindled rats to AEDs compared to other animal models (Loscher, 1997), might, therefore, be partly due to the anatomical location of the seizure focus.

The suggested ability of seizures to induce P-gp expression is consistent with clinical observations that a high number or frequency of seizures before treatment, and a long duration of seizures are associated with poorer response to AEDs (Table 8). No sustained changes in P-gp expression were produced by focal laser application to the cortex. However, altered P-gp expression has been noted in cortical dysplasia (Part III; Section 1.3) and primary brain tumours in man, and after focal brain ischaemia in animals (Part III; Section 1.2.6). This suggests that the expression pattern of P-gp may also be related to the specific pathology, rather than a response to injury or insult in general.

The relevance of these findings to epilepsy is underlined by the suggestion that several AEDs may be substrates of P-gp, raising the question of whether other drug transporters at the BBB may also limit cerebral penetration of AEDs.

8.2 Conclusion

While P-gp may contribute to "refractoriness" of epilepsy in a "universal" manner via seizure-induced expression, it may also have a region- and pathology-specific effect. These effects might contribute to the particularly drug resistant nature of MTS-related seizures. Results from these pilot studies have generated testable hypotheses regarding the potential role of P-gp in epilepsy. Laboratory experiments to examine these issues further have been discussed in the individual sections. To test the hypothesis clinically, seizure control may be compared between patients treated with and without a P-gp inhibitor (e.g. dipyridamole) as an adjunct to a P-gp substrate AED, the best demonstrated of which appears to be TPM.

OVERALL DISCUSSION AND CONCLUSION –

PREVENTION OF REFRACTORY EPILEPSY: A HYPOTHESIS

Based on an improved knowledge of the natural history of treated epilepsy derived from clinical data, including those described in this thesis, and accumulated laboratory findings, a hypothesis for the conceptual understanding and prevention of refractory epilepsy may be formulated.

Refractory epilepsy as a distinct condition

Although the criteria for defining "refractory epilepsy" are elusive e.g. number of drugs tried, dose of drugs, duration of treatment, etc (Perucca, 1998; Regesta and Tanganelli, 1999), a hard-core of 20-30% patients have a seizure disorder that appears to be resistant to all pharmacological manipulations (Sander, 1993). These patients are often treated with multiple AEDs, which, in combination, may produce sedative and behavioural toxicity (Vermeulen and Aldenkamp, 1995; Drane and Meador, 1996). High seizure frequency, prolonged seizures and episodes of status epilepticus can lead to cognitive decline (Devinsky, 1999). A long period of imperfect seizure control produces disturbed psychosocial functioning resulting in, for instance, poor academic achievement, diminished self esteem, low rates of marriage and reproduction and unsatisfactory quality of life (Cramer, 1994; Sillanpaa et al, 1998). In addition, refractory epilepsy is associated with excess mortality, particularly due to sudden unexpected death in epilepsy (Nilsson et al, 1999). "Refractory epilepsy", therefore, may be better understood as a distinct condition comprising of a constellation of disabilities with recurrent seizures being only one of its manifestations (Table 29).

Table 29. Refractory epilepsy as a distinct condition with multiple dimensions.

Intractable seizures
Excessive drug burden
Neurobiochemical plastic changes
Cognitive decline
Psychosocial dysfunction
Dependent behaviour
Restricted lifestyle
Unsatisfactory quality of life
Increased mortality

Refractory epilepsy as a multifactorial condition

Important insights into the mechanisms underlying the genesis of a range of epilepsy syndromes are emerging (McNamara, 1999), but the biological basis of "refractoriness" has not been well studied. Potential candidates, including the underlying pathology and syndromic classification, abnormal neuronal circuitry, changes in neurotransmitter receptors, ion channelopathies, reactive autoimmunity, and access of AEDs to seizure focus, were discussed in Part I; Section 5. Several of these factors were explored in the clinical and laboratory projects described in this thesis.

Consistent with previous reports, epilepsies with a known or suspected cerebral abnormality were found to be more likely to remain uncontrolled than idiopathic (genetic) epilepsies (Part II; Section 1). Among the former group of patients, seizures related to MTS were most drug-resistant (Part II; Section 4). Raised titres of autoantibodies against GAD were found in a small proportion of patients with uncontrolled epilepsy (Part II; Section 5). Their relationship to refractoriness, however, is unclear. Animal experiments suggested that P-gp at the BBB limits brain access of certain AEDs (Part III; Section 2). The hippocampus appears to over-express P-gp, potentially contributing to the particular refractoriness of focal epilepsy associated with HS / MTS (Part III; Section 3). P-gp expression can be detected in brain tissues resected from patients with refractory temporal lobe epilepsy (Part III; Section 7). The pathogenesis of refractory epilepsy, therefore, is likely to be multifactorial and variable.

Refractory epilepsy as a progressive condition

There is an overwhelming body of experimental evidence to support the concept that seizures may be self-perpetuating. The most thoroughly studied substrate is that arising

from the temporal lobe (Sutula and Hermann, 1999). Recurrent seizures, particularly those involving the limbic structures, are recognised to cause enduring disturbances in neuronal function independent of the underlying pathology (Glass and Dragunow, 1995; Liu et al, 1995; Blümcke et al, 1999). The characteristic pattern of neuronal loss and mossy fibre sprouting associated with human temporal lobe epilepsy can be produced by experimental seizures in animals (Meldrum et al, 1973; Sutular et al 1994; Babb, 1999; Coulter, 1999). Synaptic reorganisation of the mossy fibre axons of hippocampal dentate granule cells into the inner molecular layer of the dentate gyrus (Sutula et al, 1988) can form functional recurrent excitatory synapses which, in the presence of reduced inhibition, could promote seizures (Bausch and McNamara, 1999). Whether a brief seizure or even a cluster of events is sufficient to destroy neurones is less certain, but the degree of neuronal loss in susceptible regions of the hippocampus has been shown to correlate with the number of kindled-seizures (Cavazos et al, 1994). Even a single kindled seizure can induce apoptotic cell death in the dentate gyrus (Bengzon et al, 1997).

Long-lasting changes in excitatory and inhibitory neurotransmitter receptors and ion channels have been documented in these animal models of human epilepsy (Mody, 1999; Prince, 1999). Seizures are also recognised to trigger a cascade of gene expression including the immediate early genes (e.g. *c-fos*; Burazin and Gundlach, 1996), followed by neurotrophic factors (Bausch and McNamara, 1999), and a variety of late genes encoding a range of peptides, receptors, cytoskeletal proteins etc (Cole, 2000). Even a single seizure can produce prolonged increase in P-gp expression (Part III; Section 5).

The functional consequences of such neuronal plasticity remains to be fully elucidated, but these alterations may lead to a deleterious combination of disinhibition and

hyperexcitability (Babb, 1999; Wasterlain et al, 1999). This process may also account for the phenomenon of secondary epileptogenesis, which is well described in animal models of focal epilepsy. In this scenario, a primary epileptogenic lesion is able to induce epileptiform behaviour in initially normal cell populations via recurrent discharges to create a "mirror focus" which, in time, becomes an independent source of epileptic activity (Morrell, 1991).

Are there data to support such self-perpetuation in human epilepsy? Hippocampal neuronal loss following prolonged febrile seizures (Vanlandingham et al, 1998) and progressive hippocampal atrophy in refractory temporal lobe epilepsy (O'Brien et al, 1999) have been documented *in vivo* by serial magnetic resonance imaging. Other recent neuroimaging studies have reported a correlation of increasing duration of epilepsy and / or numbers of seizures with more severe neuronal loss and dysfunction in temporal lobe epilepsy (Van Paesschen et al, 1997b; Kalviainen et al, 1998; Tasch et al, 1999), although the results are not as uniform as the laboratory data (Sutula and Hermann, 1999). A prolonged history of seizures prior to epilepsy surgery is associated with poorer outcome, suggesting the development of secondary epileptogenesis as a result of recurrent seizures in unresected tissue (Eliashiv et al, 1997). Other surgical studies have reported more severe cognitive impairment in patients with longer duration of temporal lobe epilepsy (Jokeit and Ebner, 1999). Significantly, such associations have persisted even in patients who became seizure-free after temporal lobectomy.

Whether such progression can be observed in other epilepsy syndromes has been less well studied. Electroclinical studies in patients with tumour-associated epilepsy support the operation of secondary epileptogeneis (Morrell, 1991; Lim et al, 1991). On the other hand,

population-based epidemiological studies have traditionally failed to demonstrate the self-perpetuating effect of repeated seizures, particularly in children (Part I; Section 5.1). Randomised drug studies suggest that prevention of febrile seizures or posttraumatic seizures by AEDs does not reduce the risk of later recurrence (Part I, Section 5.1), although these seizures are clearly provoked and would not fulfil the diagnostic criteria of epilepsy (Commission, 1989; 1993). In the long-term outcome study of 470 adolescents and adults with newly diagnosed epilepsy described in Part II; Section 1, 47% became seizure-free on the first AED, 13% on a second monotherapy, but only 1% on the third drug. The probability of attaining seizure-freedom declined rapidly and progressively with successive AED regimens after the first two treatment manipulations.

Lastly, population-based epidemiological studies often focus purely on seizure control without consideration of the cognitive and psychosocial aspects of epilepsy (Kwan and Brodie, 2001). There is evidence to suggest that psychosocial dysfunction may not be reversible when the seizure disorder finally comes under control (Elishav et al, 1997; Engel, 1999). Surgical outcome studies indicate that even when the operation is successful in eliminating seizures, patients with a long history often do not gain employment, marry, or have children, but rather remain dependent on family and the welfare system (Engel, 1999). Such irreversibility may also explain why patients with childhood-onset epilepsy suffer social and educational disadvantages even well after entering remission in adulthood (Sillanpaa et al, 1998).

Refractory epilepsy as a preventable condition

Can these deleterious neuronal and psychosocial changes be prevented by therapeutic intervention? Although epileptogenesis is a potential target (Dichter, 1997; Clark and

Wilson, 1999) and some AEDs have demonstrated ability to retard the epileptogenic process in animal models (Loscher et al, 1998), the underlying neuropathology and the syndromic classification are at present considered to be constitutively determined. However, the other changes described above may have developed with time as a result of uncontrolled seizures. By interrupting this self-perpetuating process early, it might be possible to halt or even reverse the progression of seizure-related neurobiological, cognitive and psychosocial dysfunction.

The management of epilepsy is undergoing a revolution. Advances in neuroimaging have improved the identification of suitable candidates for epilepsy surgery, which can render up to 90% of selected groups seizure-free. The most well-defined of these are patients with MTS (Part I; Section 3.4). The timing of surgery remains controversial. Since secondary epileptogenesis at sites distant may develop with uncontrolled seizures (Eliashiv et al, 1997), early surgery to avoid deterioration in seizure control and controllability has been advocated (Engel, 1999).

Parallel to the advances in surgery, nine new AEDs have been licensed over the past decade, greatly broadening the physician's pharmacological armamentarium (Part I; Section 3). The argument against combination therapy traditionally has been its propensity to cause greater toxicity without substantial improvement in outcome (Schmidt and Gram, 1995). However, some of the newer agents may be better tolerated than their older counterparts (Brodie, 1999), potentially enhancing their overall effectiveness (Part II; Section 2). Their mechanistic diversity holds out the possibility of "rational" polytherapy (Deckers et al, 2000). "Synergistic" effects of certain combinations have been explored in laboratory and clinical studies, particularly those involving a sodium channel blocker and

an AED that enhances GABA-ergic inhibition or has multiple mechanisms of action (Part II; Section 3).

A working hypothesis

It may be hypothesised that early targetting of aggressive intervention, such as epilepsy surgery or "rational" polytherapy, may prevent the development of refractory epilepsy. A strategic plan for managing epilepsy should be formulated from the outset (Figure 40). Such a rational approach is particularly important for "high risk" patients, including those with a known or probable structural cerebral abnormality (especially certain pathologies such as MTS, CD), or other poor prognostic clinical factors such as high pre-treatment seizure numbers. Among the newly diagnosed patients studied (Part II; Section 1), those in whom the first AED failed due to lack of efficacy were four times more likely to remain uncontrolled than those who switched treatment due to intolerable side-effects. Indeed, their chance of eventual seizure-freedom was only 11%

The aim of treatment in all patients should be the maintenance of a normal lifestyle by complete seizure control with no or minimal side effects (Brodie and Dichter, 1996), given that the majority will respond to monotherapy and require lifelong treatment. The first AED should be chosen by "matching" the individual characteristics of the patients (age, gender, body habitus, concomitant disease and medications, etc), the seizure disorder (seizure type, epilepsy syndrome), and the drug (range of efficacy, tolerability, side effect and interaction profiles; Brodie and Kwan, 2001). In this regard, efficacy and tolerability should both be considered when assessing the effectiveness of an AED (Part II; Section 2).

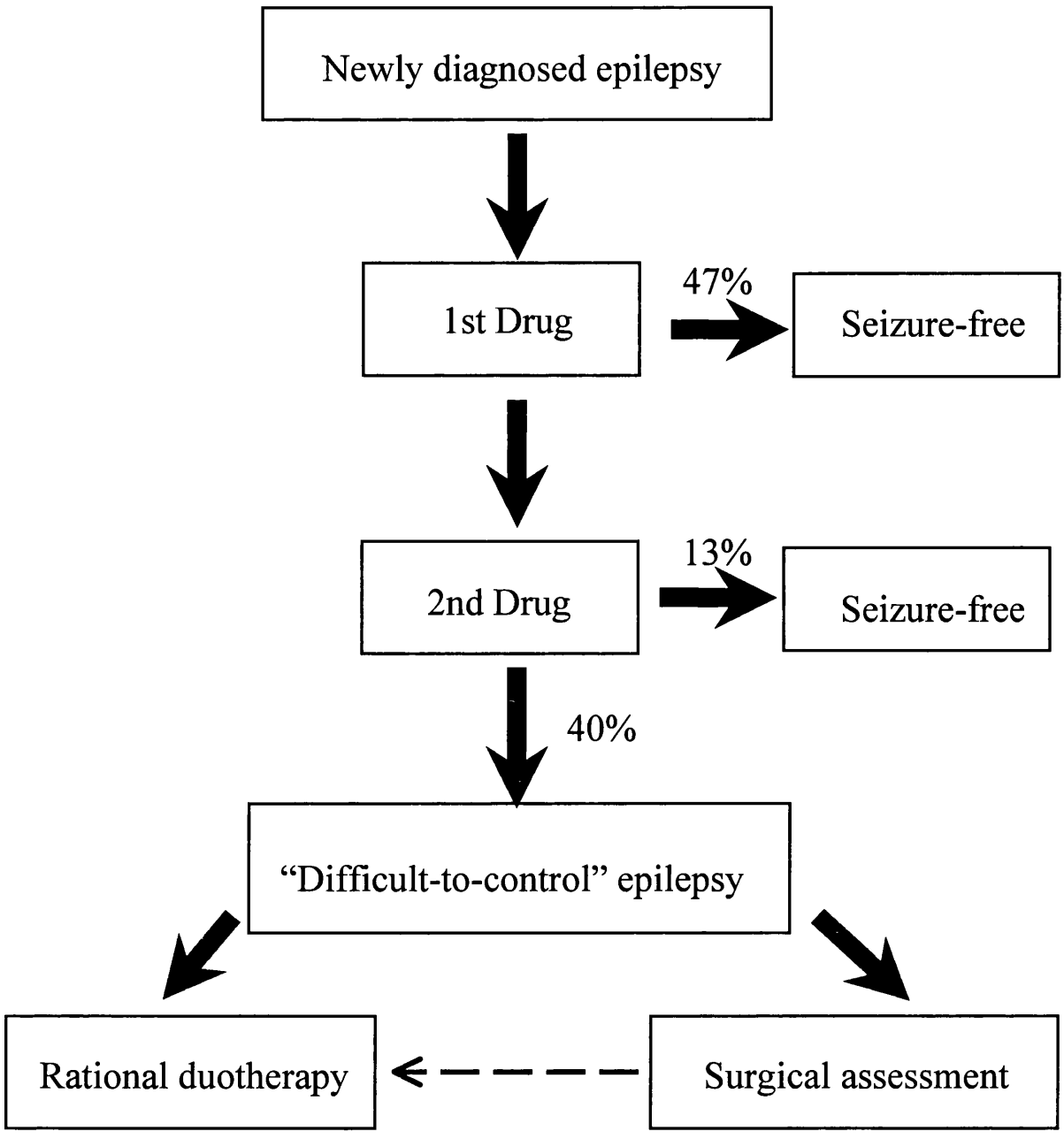


Figure 40. Strategies for managing newly diagnosed epilepsy.

If seizure control remains suboptimal despite a substantial dose of the first AED, it may not be beneficial to push on with monotherapy to the limit of tolerability since it is unlikely to be successful (Part II; Section 2). Rather, it may be more productive to substitute the original AED with one with a different mode of action or add in another with multiple pharmacological effects (Part II; Section 3). This decision will depend on the extent of the response to the first AED and the presence or absence of side effects.

Failure of two first-line AEDs due to lack of efficacy as monotherapy in a patient with correctable structural brain abnormality should prompt immediate consideration for epilepsy surgery (Part II; Section 1). For the majority of patients not suitable for "curative" resective surgery, two (or at most three) AEDs should be combined in a "rational" fashion. This should involve consideration of mechanism of action, range of efficacy, propensity for adverse interaction (pharmacokinetic and pharmacodynamic), and side effect profile (Brodie and French, 2000).

Future studies

This hypothesis may be tested at a number of levels. The main aim would be to better define the natural history of epilepsy, particularly in terms of response to AED treatment. Ideally, this should be examined in a prospective community-based setting, recruiting patients from their first seizure with pre-defined outcome measures and endpoints. To avoid selection effects due to local expertise and preference, muliple centres are required. A defined protocal, including diagnostic and classification criteria, investigation modalities, and treatment plan, would allow standardisation of the data quality.

Both cumulative and terminal remission of various lengths should be measured. Outcome measures should include not only seizure control, but also mortality and other important psychosocial aspects such as cognitive function, education attainment, employment, marriage and fertility, and other quality-of-life issues. Since drug response is heavily influenced by the underlying aetiology, subanalyses for different pathologies are required. An acturial approach should be undertaken when analysing putative risk factors.

For ethical reasons, patients with two or more unprovoked seizures should be offered an AED and a placebo arm should not be included. Specific drug choice should be left to each centre's discretion to mantain the generic nature of the results, but titration regimens need to be standardised. Whenever the AED causes intolerable adverse events despite lowering the dose, it should be substituted.

To compare the effectiveness of early and delayed combination therapy, patients with inadequate seizure control despite taking a sufficient dose of monotherapy may be randomised to substitution or addition of a second drug. To evaluate the effectiveness of a mechanistic approach, patients in the second arm may be further randomised to combinations involving a sodium channel blocker and an AED with multiple mechanisms of action, or other combinations. If the epilepsy remains uncontrolled at this stage, patients with surgically remediable lesions (such as MTS) may be randomised to undergo surgical evaluation proceeding to epilepsy surgery, or futher medical treatment for a further defined period of time. This would allow comparion between early and late surgery.

To test the "MDR hypothesis", a larger number of *mdr1a/1b* knockout mice are required to analyse across a wider range of doses and time points. Other novel AEDs should be

screened. The effect of chronic dosing should also be investigated to mimic their clinical usage more closely, and to examine whether these AEDs, similar to many other substrates, also induce the expression of P-gp. Studies to determine whether co-administration of substrate AEDs with a P-gp inhibitor may enhance their access to the brain would aid assessment of the feasibility of such "rational polytherapy" in the clinical setting. Expression of P-gp at the protein level should be measured in different brain regions to correlate with the mRNA findings. The effect of recurrent seizures and prolonged seizures (status epilepticus) on P-gp expression should also be investigated in appropriate animal models. Other seizure models, including those of focal and generalised origin, should be studied to determine whether the changes in P-gp expression are confined to GEPRs or are a consequence of seizures in general. P-gp expression in different epileptogenic pathologies (e.g. MTS, cortical dysplasia) should be compared to correlate with clinical outcome data. Ultimately, to test whether the hypothesis has relevance to patient management, seizure control may be compared between patients treated with and without a P-gp inhibitor as an adjunct to a P-gp-substrate AED.

Conclusion

Conceptualisation of refractory epilepsy as a distinct condition characterised by progressive neuronal, cognitive and psychosocial deterioration would allow a better appreciation of its multifaceted dimensions. This would support timely utilisation of powerful therapeutic interventions at an early stage in the "natural history" of treated epilepsy. P-gp, or other drug transporters, may be an important factor in the pathogenesis of refractory epilepsy in some patients. Defining its exact role may open novel therapeutic approaches to the treatment of epilepsy.

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Appendices

Appendix 1 – List of suppliers

Amersham Pharmacia Biotech, St Albans, UK
- Random hexamers

BioGene, Kimbolton, UK
- "Hi-Pure" Low EEO Agarose

CLONTECH Laboratories UK Ltd, Basingstoke, UK
- AdvanTAge PCR Cloning Kit

Life Technologies Ltd, Paisley, UK
- MMLV reverse transcriptase
- Taq DNA polymerase
- dNTP's
- 10xPCR Mg^{2+}-free buffer
- $MgCl_2$ for PCR and RT
- W-1 detergent
- Dithiothreitol

MWG-Biotech UK, Milton Keynes, UK
- Oligonucleotide PCR primers

Promega, Southampton, UK
- *PstI*
- *NcoI*
- Rnasin RNase inhibitor
- RQ1 DNase
- RNAgent Total RNA Isolation System
- SP6 RNA polymerase
- Wizard DNA Clean-Up System

QIAGEN Ltd, Crawley, UK
- QIAfilter Maxi Plasmid Kit

Sigma-Aldrich, Poole, UK
- Diethypyrocarbonate (DEPC)
- Ethidium bromide (EtBr)

Stratagene Europe, Amsterdam, The Netherlands
- NucTrap Probe Purification Column

Appendix 2 – Preparation of solutions

All chemicals were obtained from Sigma-Aldrich Co. Ltd, Poole, UK, unless otherwise stated in Appendix 1.

Electrophoresis buffer

EDTA	3.72g
dH_2O	1000ml

The pH was adjusted to 7.0 with 5M NaOH.

Tris Base	48.4g
dH_2O	5000ml

This was mixed with the EDTA solution. The pH was adjusted to 8.0 with glacial acetic acid. The buffer was made up to 10 litres with dH2O.

L-amp agar plates

LB agar tablets x 10	16.8g
dH_2O	500ml

This was autoclaved and allowed to cool to hand warm.

Ampicillin sodium (25mg/ml stock)	2ml
	502ml

This was poured into sterile 100mm plates, allowed to set at room temperature and stored at 4°C.

LB-amp medium (100µg/ml)

LB-broth	500ml
Ampicillin sodium (100mg/ml stock)	0.5ml
	500.5ml

This was stored at 4°C.

LB-broth

LB broth tablets x 10	11g
dH_2O	500ml

This was autoclaved and stored at 4°C.

DNA Loading dye

Glycerol	1ml
dH_2O	1ml
Bromophenol Blue	Pinch
Xylene Cyanole FF	Pinch

This was stored at 4°C.