# Defining the mechanistic role of High-Mobility Group Box-1 and its Utility as a Biomarker in the Inflammatory Pathogenesis of Epilepsy

Thesis submitted in accordance with the requirements of the University of

Liverpool for the Degree of Doctor in Philosophy

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This thesis is the result of my own work and the material contained within the thesis has not been presented, nor is currently being presented, either wholly, or in part, for any other degree or qualification.

Lauren Elizabeth Walker

The research presented in this thesis was carried out in the Wolfson Centre for Personalised Medicine, Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine This thesis is dedicated to my children, Rosie and Elsie, whom no matter what I do, will always be my greatest achievement.

#### Abstract

Epilepsy, which affects 50 million people worldwide, is a chronic neurological condition characterized by a predisposition to generate spontaneous seizures. Antiepileptic drug resistance is a significant problem, the causes of which are poorly understood. Inflammation is purported to play a pathological role in the development of epilepsy following brain insult. High mobility group box-1 (HMGB1) has been implicated in the development of seizures and epilepsy in preclinical models and human studies. HMGB1 undergoes post-translational modifications, including acetylation and redox changes, which dictate its inflammatory extracellular function. Novel inflammatory blood biomarkers such as HMGB1 that are intricately involved in the epilepsy disease process per se may act as stratification markers to identify patients who may benefit from immunomodulatory interventions. This thesis aimed to characterise the role of HMGB1 in seizures and epilepsy and its utility as a clinical biomarker. Analysis of 24 healthy volunteers undergoing a 24-hour blood-sampling study did not demonstrate any significant circadian fluctuations in serum HMGB1. No intra or inter-subject variability was also observed in the biomarker. A further study involving patients with idiopathic intracranial hypertension (IIH, n=18), neuroinfection (n=15) and Rasmussen's encephalitis (n=10) showed that there was no correlation between serum and cerebrospinal (CSF) fluid levels of HMGB1, regardless of blood brain barrier integrity. Subgroup analysis of bacterial meningitis showed that both CSF and serum HMGB1 was significantly elevated (as compared to IIH). Furthermore, CSF HMGB1 was more than 10-fold higher in those with bacterial (n=6) rather than viral meningitis (n=8). The expression pattern of HMGB1 acetylation and redox isoforms in brain and blood was examined in three distinct preclinical models of seizures and epilepsy including recurrent seizures and status epilepticus in the kainate-model, single seizure in the maximal electroshock test (MES) and chronic spontaneous seizures in the pilocarpine epilepsy model. In response to kainate-induced seizures, in both brain and blood, an early rise in non-acetylated and reduced HMGB1 isoforms was demonstrated consistent with functional chemotaxis. This was followed by a delayed 6-fold rise at 24 hours in brain of the acetylated, disulphide inflammatory form of HMGB1. In serum, significant expression of the inflammatory isoforms was seen after 14 days, possibly coinciding with the onset of spontaneous seizures. Inflammatory isoforms of HMGB1 were not identified within the first 24 hours following isolated MES-seizure in mice. Serum, but not brain, total HMGB1 was significantly elevated (by 311%) in chronic epileptic mice experiencing regular spontaneous seizures; however the contribution of the different isoforms remains to be elucidated. In humans with epilepsy, compared to both healthy controls (1.11±0.07ng/ml, p<0.0001) and those with well-controlled epilepsy (1.25±0.15ng/ml, p<0.0001), mean baseline total HMGB1 was significantly higher in patients with drug-resistant epilepsy (8.70 ±0.47ng/ml). Acetylated HMGB1 was observed in drug-resistant patients alone; with a subset expressing the disulphide inflammatory form. In conclusion, these studies have provided insight into the potential of novel, circulating isoforms of HMGB1 to serve as mechanistic biomarkers of established drug-resistant epilepsy in humans. There is a need for future studies to examine the prognostic value of HMGB1 isoforms following first seizure for the early identification of those at greater risk of developing drug resistance and ultimately, those who may benefit from immunomodulatory interventions.

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## Abbreviations

ADDIEVIA	
AED	Antiepileptic drug
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ASC	Apoptosis-associated speck-like protein containing a CARD
AUC	Area under the curve
BBB	Blood brain barrier
CK	Creatine kinase
CNS	Central nervous system
	Cyclooxygenase-2
CPS	Complex nartial seizure
CRP	C-reactive protein
CSE	Cerebrospinal fluid
CT	Computed Tomography
	C X C matif chamaking 12
CXCD4	CYC recentor type 4
	CAC-receptor type 4
DAIVIP	Damage associated molecular pattern
DC	
DNA	Deoxyribonucieic acid
EEG	
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FDR	False discovery rate
GABA	Gamma-aminobutyric acid
GFAP	Glial fibrillary acidic protein
GLuR3	Glutamate Receptor 3
HMGB1	High Mobility Group Box-1
ICAM1	Intercellular Adhesion Molecule-1
ICE	Interleukin converting enzyme/Caspase-1
IFCC	International Federation of Clinical Chemistry
IFNγ	Interferon-γ
IGE	Idiopathic generalised epilepsy
IHC	Immunohistochemistry
IIH	Idiopathic intracranial hypertension
ILAE	International League Against Epilepsy
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-18	Interleukin-18
IL-1RA	Interleukin-1 receptor antagonist
IL-1R1	Interleukin-1 receptor type 1
i.p.	Intraperitoneal
IVlg	Intravenous Immunoglobulin
JME	Juvenile myoclonic epilepsy
KA	Kainic acid
LCMS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LCMV	Lymphocytic choriomeningitis virus
LP	Lumbar puncture
 I PS	

LRR	Leucine-rich repeat
MAb	Monoclonal antibody
MAPKs	Mitogen-activated protein kinases
MCAO	Middle cerebral artery occlusion
MES	Maximal electroshock
MRI	Magnetic Resonance Imaging
Nalps	NACHT-, LRR-, and pyrin domain–containing proteins
NF-ĸB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	Nucleotide-binding oligomerization-domain protein-like receptor
NLRP3	NACHT, LRR and PYD-containing protein 3
NMDA	N-methyl-D-aspartate
NIH	National Institute of Health
NO	Nitric oxide
PAMP	Pathogen associated molecular pattern
PET	Positron emission tomography
PGS	Primarily generalised seizure
PIL	Patient information leaflet
PRR	Pattern recognition receptors
RAGE	Receptor for Advanced Glycation End products
RE	Rasmussen's encephalitis
RIPA	Radio-Immunoprecipitation Assay
ROC	Receiver Operator Characteristic
SAH	Subarachnoid haemorrhage
SD	Standard deviation
SE	Status epilepticus
SEM	Standard error of the mean
SGTCS	Secondarily generalised tonic clonic seizure
SPS	Simple partial seizure
SSSE	Self-sustained status epilepticus
TBS	Tris-buffered saline
TGFβ	Transforming growth factor-β
TLE-HS	Temporal lobe epilepsy with hippocampal sclerosis
TLR	Toll-like receptor
τνγα	Tumour necrosis factor α
TSS	Transverse cerebral venous sinus stenosis
VCAM1	Vascular cell adhesion molecule-1
VGCC	Voltage-gated calcium channel
VGKC	Voltage-gated potassium channel
WB	Western blot

## Publications arising as a result of this thesis:

- 1. Walker LE, Mirza N, Yip VL, Marson AG, Pirmohamed M. Personalized medicine approaches in epilepsy. *J Intern Med*. 2015;277(2):218-34
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- Walker L, Pirmohamed M, Marson AG. Immunomodulatory interventions for focal epilepsy syndromes. *Cochrane Database Syst Rev.* June 2013 DOI: 10.1002/14651858.CD009945.pub2
- 4. **Walker L**, Sills GJ. Inflammation and epilepsy: the foundations for a new therapeutic approach in epilepsy? *Epilepsy Curr.* 2012; 12(1):8-12.

## Abstracts

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# **General Introduction**

#### 1.1 Epilepsy

Epilepsy is a chronic neurological condition characterized by an enduring predisposition to generate spontaneous epileptic seizures (Fisher et al., 2005) affecting approximately 50 million worldwide (Leonardi and Ustun, 2002). Diagnosis requires the occurrence of at least one epileptic seizure but also recognises the "neurobiological, cognitive, psychological and social consequences" of the condition (Fisher et al., 2005). The consequences of a persistent abnormality of the brain are far reaching and include significant morbidity and mortality with unique stigmatization of affected individuals and a high societal cost (~15 billion/year in Europe (Pugliatti et al., 2007)).

#### 1.1.1 Seizure

The point at which an individual brain generates a seizure is termed the seizure threshold. The threshold is a dynamic concept that fluctuates normally over time as a consequence of physiological variables including circadian rhythmicity and specifically in females, the menstrual cycle (termed catamenial seizures). Anything that lowers the seizure threshold beyond a critical point will induce the clinical expression of a seizure. Epileptogenic abnormalities that lower the seizure threshold include alterations at the molecular, anatomical or circuit level and can be as a consequence of genetic, structural or metabolic anomalies. A number of other factors can further lower the seizure threshold to make the occurrence of a seizure more likely; these include stress, sleep deprivation, intercurrent infection, alcohol and various medications.

Seizures can either be generalized or focal in origin. Generalized seizures are defined as "originating at some point within, and rapidly engaging, bilaterally distributed networks (Berg et al., 2010)." In contrast, focal seizures are those "originating within networks limited to one cerebral hemisphere" (Berg et al., 2010). In their 2010 report (Berg et al., 2010), The International League Against Epilepsy (ILAE) commission on Classification and Terminology, 2005–2009, revised the existing classification of seizures (1981), table 1.1.

Table 1.1 International League Against Epilepsy Classification of seizures (2010)

Generalized seizures (originating at some point within, and rapidly engaging, bilaterally distributed networks.)
Tonic Clonic (in any combination)
Absence
Typical
Atypical
Absence with special features
Myoclonic absence
Eyelid myoclonia
Myoclonic
Myoclonic
Myoclonic atonic
Myoclonic tonic
Clonic
Tonic
Atonic
Focal Seizures (originating within networks limited to one hemisphere.)
Epileptic spasms

Taken from the International League Against Epilepsy (ILAE) commission on Classification and Terminology, 2005–2009, reproduced from (Berg et al., 2010)

A notable modification to the 1981 classification system was the elimination of the distinction in focal seizures between complex partial and simple partial, as pertains to consciousness/awareness. The new system instead recognises descriptors of focal seizures, and a glossary for descriptive terminology of ictal semiology is available (Blume et al., 2001). In brief, "simple partial" has been replaced by "without impairment of consciousness or awareness". There may be observable motor or autonomic components with or without subjective sensory or psychic phenomena. The latter corresponds to the previous concept of an aura. "Complex partial" is now described as "with impairment of consciousness or awareness" or alternatively, "dyscognitive" (Blume et al., 2001). Lastly, the term "secondarily generalized" has been replaced by "evolving to a bilateral convulsive seizure."

All currently available antiepileptic drugs (AEDs) exert their effect through raising the seizure threshold; therefore, despite the "common" nomenclature; they are in fact purely symptomatic and exhibit no disease-modifying potential. Disease modifying drugs are therefore needed and may have the potential to prevent and/or treat resistant forms of epilepsy.

## 1.1.2 ILAE Classification of epilepsy

The epilepsies are a complex group of disorders and classification is in evolution within the epilepsy community. In clinical practice, diagnosis of epilepsy follows a two-step process: 1) classification of seizure type and 2) assignment of cause (Shorvon, 2011). Advances in imaging and molecular chemistry have led to a greater understanding of the aetiopathological basis of many of the epilepsies. As such, The ILAE has recently proposed a new approach to the nomenclature. The new approach takes into account the underlying aetiology, categorising epilepsy on the basis of causality into genetic, structural/metabolic and unknown (Scheffer, 2010). The structural or metabolic category may in future become further subdivided to include immune and infectious causes but this remains under discussion (Scheffer, 2010). The classification terms and their concepts are described as:

- Genetic: Epilepsy occurs as a result of known or presumed genetic defect(s). The evidence for the genetic basis may arise from appropriately designed family studies or from specific molecular genetic studies that have been well replicated. Spontaneous seizures are at the core of the disorder (Berg et al., 2010).
- 2. Structural/metabolic: Where a substantially increased risk of epilepsy has been shown, in appropriately designed studies, to occur in association with a distinct structural or metabolic condition or disease (Berg et al., 2010). This includes acquired disorders such as head trauma, stroke and cortical malformations that may have an underlying genetic basis (for example tuberous sclerosis.)
- Unknown: "The nature of the underlying cause is as yet unknown (Berg et al., 2010)."

However, some aspects of this new approach, in particular the need for replacing the old terms idiopathic, symptomatic and cryptogenic (1989), have faced widespread disagreement in the epilepsy community (Wolf, 2010; Ferrie, 2010; Guerrini, 2010). In the original classification system, when epilepsy occurred as a result of an identifiable

brain defect, it was termed "symptomatic epilepsy." There are a large number of potential causes for this including malformations of brain development, brain trauma (including stroke, cerebral metabolism and head injury) and infections of the central nervous system (CNS). By far the commonest form of symptomatic epilepsy, which would now be considered to be structural/metabolic (with the exception of the genetic forms that have been described), is symptomatic medial temporal lobe epilepsy (TLE) characterized by complex partial seizures arising from regions within the temporal lobe, usually the hippocampus or amygdala (Chang and Lowenstein, 2003, Bertram, 2009). It is frequently, but not always, associated with a characteristic lesion involving selective neuronal loss in the CA1/CA3 region of the hippocampus and hilus, termed hippocampal sclerosis. When an identifiable cause or structural lesion could not be identified but was assumed likely to be present the epilepsy was termed 'cryptogenic'. The term 'idiopathic' was used to denote epilepsies with an assumed genetic aetiology. The original 1989 document broadly define idiopathic epilepsy as having no underlying cause, with the exception of a possible hereditary predisposition, and characterised them by age-related onset with specific clinical and electrographic parameters.

The 2010 approach also introduced the concept of "electroclinical syndromes" wherein diagnosis reflects a cluster of electroclinical characteristics, which can be organized by typical age of onset (table 1.2) (Berg et al., 2010).

## 1.1.3 Epileptogenesis

Epileptogenesis describes the process by which a normal brain is functionally altered, as a consequence of a brain insult, into one able to generate abnormal electrical activity culminating in a spontaneous seizure (Rakhade and Jensen, 2009). Ictogenesis describes the propensity of the brain to generate spontaneous seizures. The mechanisms underlying epileptogenesis are likely multifactorial given the wide variety of aetiologies that lead to epilepsy. In adulthood, epileptogenesis occurring as a consequence of traumatic brain injury (Haltiner et al., 1997; Temkin, 2001; Garga and Lowenstein, 2006) or stroke has been well described (Hauser et al., 1993). It is a process that may evolve over many years following a brain insult before the appearance of the first spontaneous seizure and there may be a sequence of varying mechanisms over this time course (Haltiner et al., 1997).

# Table 1.2 International League Against Epilepsy electroclinical syndromes arranged by age of onset

## **Neonatal Period**

Benign familial neonatal epilepsy (BFNE)
Early myoclonic encephalopathy (EME)
Ohtahara syndrome
Infancy
Epilepsy of infancy with migrating focal seizures
West syndrome
Myoclonic epilepsy in infancy (MEI)
Benign infantile epilepsy
Benign familial infantile epilepsy
Dravet syndrome
Myoclonic encephalopathy in non-progressive disorders
Childhood
Febrile seizures plus (FS+) (can start in infancy)
Panayiotopoulos syndrome
Epilepsy with myoclonic atonic (previously astatic) seizures
Benign epilepsy with centrotemporal spikes (BECTS)
Autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE)

Late onset childhood occipital epilepsy (Gastaut type)

Epilepsy with myoclonic absences

Lennox-Gastaut syndrome

Epileptic encephalopathy with continuous spike-and-wave during sleep (CSWS)

Landau-Kleffner syndrome (LKS)

Childhood absence epilepsy (CAE)

## Adolescent- Adult

Juvenile absence epilepsy (JAE) Juvenile myoclonic epilepsy (JME) Epilepsy with generalized tonic–clonic seizures alone Progressive myoclonus epilepsies (PME) Autosomal dominant epilepsy with auditory features (ADEAF) Other familial temporal lobe epilepsies

## Less specific age relationship

Familial focal epilepsy with variable foci (childhood to adult) Reflex epilepsies

Modified from (Berg et al., 2010).

Seizures in early life may be triggered by external insults including prolonged febrile convulsions (Shinnar et al., 2008), trauma (Dinner, 1993) and hypoxic-ischaemic encephalopathy (Volpe, 2008) which, which may then be following delayed epileptogenesis, leading to the development of epilepsy in later life.

Unravelling the mechanisms of epileptogenesis will involve first identifying whether different aetiologies trigger identical, overlapping or distinctly unrelated pathways leading to an abnormal epileptic focus.

## 1.1.4 Pharmacotherapy

Treatment of epilepsy began in 1857 (figure 1.1) with the use of potassium bromide in women with "hysterical epilepsy connected with the menstrual period." This was followed by the hypnotic agent phenobarbital, its anticonvulsant properties discovered by chance by Alfred Hauptmann in 1912, and still the most commonly prescribed AED in the developing world (Kwan and Brodie, 2004). Phenytoin was next; clinically evaluated in 1936, the first patient to take it was rendered seizure-free having experienced daily seizures for many years prior to its introduction (Merritt HH, 2004). Carbamazepine, sodium valproate and the benzodiazepines did not appear until the 1960's followed by the modern era of AED development beginning in 1975 with the establishment of the National Institute of Health (NIH) antiepileptic drug development programme.

In the last 15 years alone the number of licensed drugs has more than doubled (table 1.3). The post-1993 era of AED drug development has offered considerable improvement in terms of safety, improved tolerability and favourable pharmacokinetics (table 1.4) (Bialer, 2006; Bialer et al., 2007; Bialer et al., 2009). Despite the welcome advantages of newer AEDs which avoid adverse drug interactions and hypersensitivity reactions (Elger and Schmidt, 2008), their overall efficacy in new onset epilepsy is no better than that of the older AEDs (Kwan and Brodie, 2000; Marson et al., 2007c; Glauser et al., 2010; Brodie et al., 2007). In fact, despite a therapeutic arsenal of 24 drugs and 1 medical device, concern is growing that the efficacy of epilepsy therapeutics has not substantially improved in the last 20 years (Shorvon, 2009).



Figure 1.1 The introduction of antiepileptic drugs into the market from 1857 until 2009. The year given is that of first licensing, or the first mention of clinical use in Europe, the United States or Japan. Reproduced with permission from (Loscher and Schmidt, 2011), adapted from the original (Shorvon, 2009a).

The SANAD (Standard and New Antiepileptic Drug) trial was designed to assess the clinical and cost effectiveness of new AEDs compared to standard AEDs and was divided into two arms (Marson et al., 2007c). Arm A compared new drugs lamotrigine, gabapentin, topiramate or oxcarbazepine with the existing first-line agent carbamazepine. In terms of 12-month remission from seizures, none of the new drugs proved superior in efficacy to carbamazepine. Lamotrigine was found to be non-inferior to carbamazepine (Marson et al., 2007a), whilst gabapentin and topiramate were inferior. Arm B studied lamotrigine and topiramate versus valproate for the treatment of generalised and unclassified epilepsy. The study showed a non-significant advantage of valproate over topiramate for time to 12 month remission, and that topiramate was significantly more likely to be withdrawn, mainly due to poorer

tolerability. Valproate was significantly superior to lamotrigine for time to 12 month remission (Marson et al., 2007b).

Before 1993	1993-2005	2009-2011	
Carbamazepine	Felbamate	Vigabatrin	
Clonazepam	Gabapentin	Rufinamide	
Diazepam	Lamotrigine	Lacosamide	
Ethosuximide	Levetiracetam	Clobazam	
Lorazepam	Oxcarbazepine	Ezogabine	
Phenobarbital	Pregabalin		
Phenytoin	Tiagabine		
Primidone	Topiramate		
Valproic acid	Vagus nerve stimulation		
	Zonisamide		

Table 1.3 Antiepileptic drug	s and	devices	currently	approved	by the	Food	and	Drug
Administration								

Reproduced from (Sirven et al., 2012)

Table 1.4 Pharmacokinetic properties of some of the commonly prescribedantiepileptic drugs

Drug	Plasma protein binding (%)	Time to steady state (days)	Dosing frequency	Initial monitoring frequency (weeks)
Carbamazepine	70-80	3-10	bid, tid, qid	3,6 or 9
Phenytoin	90	5-15	qd or bid	2-3
Valproic acid	60-95	2-4	bid or tid	1-2
Lamotrigine	50-55	5-15	bid	none
Levetiracetam	<10	3-4	bid	none
Pregabalin	0	2	bid, tid	none
Gabapentin	0	1-2	tid	none
Topiramate	9-17	5-7	bid	none
Zonisamide	40-60	12-14	qd, bid	none

Adapted from (Schachter, 2007). Bid (bis in die) twice a day; tid (ter in die) three times daily; qd each day; qid four times daily.

Valproate remains the drug of first choice for generalised epilepsies, with the exception of women of childbearing potential due to the risks of teratogenicity and neurodevelopmental problems (Bromley et al., 2014).

## **1.2** Epilepsy and drug resistance

Despite the availability of many different anti-epileptic drugs, approximately one third of patients with epilepsy continue to experience seizures (Cockerell et al., 1995).

In most cases, the reasons for this drug-resistance remain unknown. The spectrum of drug resistance is very wide, ranging from mild seizures with partial resistance to frequent, severe seizures unresponsive to multiple medications. The ILAE defines drug resistance as "failure of adequate trials of two tolerated and appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom (Kwan et al., 2010)."

## **1.2.1** Patterns of drug resistance

Three distinct patterns of drug-resistance in epilepsy have been suggested on the basis of epidemiological evidence:

- 1. *De novo drug resistance:* Wherein patients exhibit drug-resistance prior to commencing AED therapy. It has been shown that, in patients in whom the first AED failed to achieve seizure control, the probability of future success with an alternative AED was only 11%, as compared to 41-55% in patients who ceased therapy for an alternative reason (e.g. idiosyncratic reactions) (Kwan and Brodie, 2000).
- 2. *Progressive drug resistance:* Wherein resistance appears following an initial period of good control. This has been reported in some childhood epilepsies and mTLE (Berg et al., 2006).
- 3. *Waxing and waning resistance:* Wherein intractability is not sustained. Patients may alternate between responsiveness and resistance to AEDs, possibly related to changes in drug bioavailability and tolerance to AEDs (Loscher and Schmidt, 2006). A prospective study recently identified 5% per year of patients initially deemed to be drug-resistant enter seizure remission. However, a substantial proportion of these patients then relapse within a year (Callaghan et al., 2011).

For many, surgical resection of the epileptic focus remains the only available treatment option however; many patients are unsuitable surgical candidates. Both the site of seizure origin and the possible post-surgical neurological outcomes must be rigorously evaluated, at considerable expense. Complicating matters, most AEDs exhibit dosedependent adverse effects which result in up to 25% of patients discontinuing treatment for intolerable side effects prior to attaining a fully-effective dosage (Kwan and Brodie, 2000; Perucca et al., 2009).

## **1.2.2** Theories of drug resistance

Currently, three main theories exist to explain drug resistance in epilepsy. These can be divided into the drug transporter hypothesis, the drug target hypothesis, and the inherent severity model of epilepsy.

#### **1.2.2.1** The drug transporter hypothesis

The most popular and extensively studied theory of drug resistance in epilepsy has been the multidrug transporter (MDT) hypothesis (Chayasirisobhon, 2009). This hypothesis states that drug penetration into the epileptic focus is impaired due to dysregulation of drug transporters (Chayasirisobhon, 2009). The MDTs involved in drug resistance in humans are either adenosine triphosphate-binding cassette (ABC) proteins (Tiwari et al., 2011) or SLC proteins (Huang and Sadee, 2006). Research efforts have focused predominantly on a single ABC transporter, P-glycoprotein (P-gp; also known as ABCB1). P-gp was first suspected to be involved in drug-resistance following the finding that expression was found to correlate with drug-resistance to cancer chemotherapy in Chinese hamster ovary cells in 1979 (Riordan and Ling, 1979). It was not until 1995 that it was shown to be increased in brain tissue taken from the epileptic foci of patients with drug-resistant epilepsy (Tishler et al., 1995). Since then, evidence from both experimental "proof of principle" and clinical studies supports overexpression of P-gp in varying pathologies associated with drug-resistant epilepsy, including focal malformations of cortical development, hippocampal sclerosis and tuberous sclerosis (Aronica et al., 2012). However, for overexpression of P-gp to be the causative factor in drug-resistance, then many (if not all) AEDs would be required to be substrates. Yet despite years of intense research in this area, there remains a lack of convincing evidence that AEDs are high-affinity substrates for P-gp. (Dickens et al., 2013; Loscher and Sills, 2007; Anderson and Shen, 2007; Rogawski, 2013). Multiple individual genetic association studies and at least three meta-analyses (Leschziner et al., 2007; Bournissen et al., 2009; Haerian et al., 2010) have not replicated an

association between ABCB1 polymorphisms and epilepsy drug resistance. As such, it is difficult to see how P-gp overexpression can satisfactorily explain drug-resistance in epilepsy and hence alternative theories are still sought.

#### **1.2.2.2** The drug target hypothesis

The drug target hypothesis proposes that intrinsic (genetic) and acquired (diseaserelated) alterations to the structure and/or functionality of AED targets in epileptogenic brain regions lead to reduced drug effects (Loscher and Potschka, 2005). Intrinsic alterations confer an inherited, inborn difference in the target that confers resistance. A common functional polymorphism in the SCN1A gene, which encodes an isoform of voltage-activated sodium channels, has been associated with responsiveness to the sodium channel blockers carbamazepine and phenytoin. In Japanese patients with epilepsy, the frequency of the AA genotype was significantly higher in those resistant to carbamazepine [odds ratio (OR) 2.7, 95% confidence interval (CI) 1.1-7.1; P = 0.04] (Abe et al., 2008). Similarly, in English and Chinese patients, the AA genotype was associated with a requirement for the maximum doses of carbamazepine and phenytoin (Tate et al., 2005; Tate et al., 2006). This genotype has also been associated with changes in cortical excitability (Menzler et al., 2014): in a study of 92 healthy volunteers receiving 400 mg carbamazepine, GG homozygous subjects (at rs3812718) showed increased cortical inhibition compared to AA homozygous subjects, suggesting that rs3812718 may be modulating the response via gamma-aminobutyric acid (GABA)ergic cortical interneurons (Abe et al., 2008; Tate et al., 2005).

In the acquired form of the drug target hypothesis, the change in the target occurs in conjunction with epileptogenesis, as a result of seizures, or as a consequence of drug treatment. Neurobiological support for this hypothesis comes from preclinical studies using rat models of epilepsy. In kindled rats, using an electrical stimulus to induce an epileptic focus, sodium channels were found to exhibit reduced sensitivity to carbamazepine which normalised 5 weeks after kindling, indicating that the changes were related to the kindling and not the epileptic state (Vreugdenhil et al., 1998; Vreugdenhil and Wadman, 1999). Secondly, loss of hippocampal inhibitory neurotransmitter function has been shown in a rat model of temporal lobe epilepsy

resulting from alterations in the subunit composition of GABAA receptors (Brooks-Kayal et al., 1998). What is more, resistance to benzodiazepines has been shown to develop during prolonged status epilepticus as a result of internalization of synaptic GABAA receptors (Wasterlain and Chen, 2008; Joshi and Kapur, 2012; Fritsch et al., 2010).

The drug target hypothesis has also encountered detractors. Many studies have failed to replicate an association between *SCN1A* polymorphisms and response to either carbamazepine or phenytoin (Table 1.5); therefore, the clinical validity of this pharmacogene in the vast majority of patients with acquired epilepsies remains uncertain.

Perhaps more importantly, most patients with drug-resistant epilepsy are resistant to all AEDs. AEDs are known to have different mechanisms of action and drug targets, including subunits of voltage-gated sodium and calcium channels as well  $\alpha 2\delta$  proteins that are associated with calcium channels, GABAA receptors, the GAT-1 GABA transporter, the GABA catabolic enzyme GABA transaminase, KV7/KCNQ/M potassium channels, the synaptic vesicle protein SV2A, and  $\alpha$ -amino-3-hydroxy-5-methyl-4isoxazolepropionic acid (AMPA) receptors (Meldrum and Rogawski, 2007). For the target hypothesis to be the unifying explanation for resistance then targets of all AEDs would need to be simultaneously modified, which seems improbable, particularly given the resistance seen to even the newer generation of AEDs which have novel and distinct molecular targets.

### **1.2.2.3** The inherent severity hypothesis

The inherent severity hypothesis proposes that there is a continuum in severity of the disease, which determines its relative response to medication (Rogawski, 2013). This follows the finding, consistently shown in prospective studies of newly-diagnosed epilepsy, that the single most important factor in determining medication response is the frequency of seizures in the early phase. Put simply, the easier the seizures are to trigger, the more frequently they will occur and the more difficult they will be to suppress. It has been consistently found that having multiple seizures prior to diagnosis is a risk factor for drug resistance, and this is likely to be correlated with

epilepsy type as well as intrinsic severity (Kwan and Brodie, 2000; Hitiris et al., 2007; Berg et al., 2001; MacDonald et al., 2000; Dlugos et al., 2001).

Polymorphism	Subjects	Response	Ref.
<i>SCN1A</i> rs3812718 (G>A)	92 healthy European volunteers given 400mg carbamazepine	GG homozygotes increased carbamazepine-induced cortical inhibition than AA homozygous subjects	(Menzler et al. <i>,</i> 2014)
<i>SCN1A</i> rs3812718 (G>A)	228 Japanese epilepsy patients	Frequency of the AA genotype was significantly higher in carbamazepine-resistant patients	(Abe et al., 2008)
SCN1A rs590478, rs8191987, rs3812718, rs2126152	British epilepsy patients (425 carbamazepine/281 phenytoin)	AA genotype required maximum doses of carbamazepine or phenytoin	(Tate et al., 2005)
<i>SCN1A</i> rs3812718 (G>A)	168 Taiwanese epilepsy patients	AA genotype required higher maintenance dose of phenytoin than GG	(Tate et al., 2006)
Polymorphisms in <i>SCN1A</i> (including rs3812718), SCN2A and SCN3A genes	1504 epilepsy patients from Malaysia and Hong Kong	No significant allele, genotype and haplotype association of polymorphisms in the SCN1A, SCN2A, and SCN3A genes with drug responsiveness	(Haerian et al., 2013)
27 tagging SNPs of SCN1A (including rs3812718), SCN2A, and SCN3A.	471 Chinese epilepsy patients	No association between rs3812718 and drug responsiveness	(Kwan et al., 2008)
<i>SCN1A</i> rs3812718 (G>A)	485 North Indian epilepsy patients	No association between rs3812718 and drug responsiveness	(Kumari et al., 2013)

Table 1.5 Sodium channel, voltage-gated type I alpha subunit (SCN1A) gene polymorphisms and response to anti-epileptic drugs in epilepsy

SCN1A, sodium channel, voltage-gated, type I, alpha subunit; SCN2A, sodium channel, voltage-gated, type II, alpha subunit; SCN3A, sodium channel, voltage-gated, type III, alpha subunit; ref, reference; SNP, single nucleotide polymorphism.

Patients reporting more than ten seizures prior to initiation of therapy are more than twice as likely to develop drug-resistant epilepsy than those with two or less pretreatment seizures (Hitiris et al., 2007; Mohanraj and Brodie, 2006). However, a theory based wholly on early seizure frequency has also been challenged. Some patients with initially drug-resistant epilepsy do go on to achieve seizure freedom when treated with newer generation AEDs (Brodie, 2010; Luciano and Shorvon, 2007; Callaghan et al., 2007). In contrast, some patients with infrequent seizures are drug-resistant. However, seizure-frequency should not be considered the only measure of disease severity. A person experiencing infrequent generalised seizures may be considered to have more severe epilepsy than a patient experiencing regular simple partial seizures that do not impact on activities of daily living. What is more, infrequent seizures can be misinterpreted for seizure remission (French, 2006). However, extending the classification of remission from 1 year to 5 years seizure-free does not change the outcome in terms of association of seizure frequency with chance of remission (MacDonald et al., 2000).

Central to the intrinsic severity hypothesis is that dysfunction of neurobiological processes underlies the development of drug resistance. A prospective study following children with medial temporal lobe epilepsy (mTLE) from diagnosis showed that it was magnetic resonance imaging (MRI) lesions, and not seizure frequency, that predicted outcome (Spooner et al., 2006). The role of structural lesions in predicting resistance is further supported by the success of respective epilepsy surgery, irrespective of seizure frequency at onset (Schmidt and Loscher, 2005). However, the neurobiological processes underlying epileptogenesis remain poorly understood. To date no molecular genetic studies have been performed in patients to compare those with low, versus high, seizure frequency at presentation (Rogawski, 2013). A genome-wide analytical approach could help to identify the central causal mechanisms that would help to support this hypothesis.

## **1.2.3** Predicting drug resistance

No single clinical factor has been found to be uniquely useful in accurately predicting drug resistance at diagnosis. Prognostic modelling of data from the largest randomized open-label trial of newly diagnosed epilepsy demonstrated that 12-month remission from seizures was less likely in (i) female patients, (ii) in adolescents and young adults (ages 11–36 years) and those with (iii) neurological insults, (iv) a high number of

seizures before starting treatment and (v) a short time between first seizure and treatment initiation (Bonnett et al., 2012). The recent French ESPERA study of polypharmacy in focal epilepsy demonstrated that the mean annual direct cost was 2.3-times higher in drug-resistant than in drug-responsive patients (€4485 vs. €1926, P < 0.0001). The increased costs were largely attributed to the drugs used, and increased duration of hospitalization and number of investigations (de Zelicourt et al., 2014). The consequences of drug resistance can be severe, with mortality rates increased four- to sevenfold compared to drug-responsive patients (Sperling, 2004).

This represents a major unmet clinical need. New anti-seizure treatments for epilepsy are unlikely to bridge this treatment gap and the next generation of therapies needs to possess disease-modifying properties. Such drugs could potentially be used to halt or reverse the progression of epilepsy in people with an established diagnosis or to delay or prevent the onset of epilepsy in susceptible individuals. The major problem encountered when designing drugs for delivery to the brain is created by the unique structural and functional properties of the blood brain barrier (BBB) (Abbott et al., 2006). Free drug in blood must first pass the tight endothelial cells that create the mechanical barrier that is the BBB, limiting drug passage to and accumulation within the brain interstitial fluid (Abbott et al., 2006; Reese and Karnovsky, 1967). Only free drug within the interstitial fluid is able to access brain cells and their respective targets within. The alternative route of access to the brain is through the low protein cerebrospinal fluid (CSF) which surrounds the brain (and is actively secreted by the epithelium of the choroid plexus (Sakka et al., 2011)) Fluid turnover is slow, 3-4 times per day (Sakka et al., 2011). Furthermore, efflux transporters maintain low concentrations of free drug within the CNS.

Development of disease-modifying drugs for epilepsy would be greatly enhanced by the identification of one or more biomarkers that predict onset and progression of the disorder and its response to treatment; these are currently lacking. Ideally, such a biomarker should be sensitive and specific in terms of its association with prognosis and non-invasive and inexpensive in terms of the method of its measurement. It should also mirror the underlying pathophysiology of the disorder or, in this case, the mechanisms responsible for the generation and perpetuation of seizures, i.e. epileptogenesis.

## **1.3** Epilepsy seizure models

Preclinical animal models of epilepsy are heavily relied upon for the establishment of safety and efficacy of new AEDs prior to first in human trials (White, 2006). In order to move forwards with the discovery and development of new AEDs to target resistance, it is important to be confident that the preclinical animal models are fit for purpose. Animal models of seizure and epilepsy serve a variety of purposes in drug development including identifying new AEDs, to evaluate efficacy of new drugs against different types of seizure and epilepsies and for comparison to established therapies, to estimate effective plasma concentrations, to characterize preclinical efficacy in chronic administration (i.e. assessment of tolerance) and for discovery of disease modifying therapies following brain insult (Loscher, 2011). A variety of preclinical models have been described (figure 1.2). No single model can be used to evaluate all of the above described purposes. Models of acquired epilepsy, in which epilepsy or epilepsy-like conditions are induced by electrical or chemical methods in previously healthy (non-epileptic) animals, will be discussed here.

## 1.3.1 Status epilepticus

## 1.3.1.1 Rodents

Definitions of status epilepticus (SE) in mice can vary in the literature. Acute seizures are described according to the Racine scale (Racine, 1972) and are characterized as freezing and facial clonus (stage 1), masticatory movements and head nodding (stage 2), wet dog shakes (stage 3) forelimb clonus-unilateral or bilateral (stage 4) followed by rearing and falling (stage 5) (Raedt et al., 2009; Pernot et al., 2011; Mouri et al., 2008). A generally acceptable definition of SE in mice is continuous convulsive seizure activity above Racine stage 3 for a minimum of 30 minutes with incomplete recovery of responsiveness between episodes (Cavalheiro, 1995; Leite et al., 1990; Loscher, 2002). SE in rodents (rats and mice) can be induced either by electrical stimulation or by a chemoconvulsant, such as kainate or pilocarpine.



Figure 1.2 Schematic overview of some of the available models of epilepsy or epileptic seizures, adapted from (Loscher, 2011). MES: Maximal electro shock; PTZ: pentylenetetrazole; SE: Status epilepticus; BLA: basolateral amygdala kindling epileptogenesis model

## 1.3.1.2 Humans

In humans, the spectrum of SE is very wide and indeed classification of SE in humans is a subject of much discussion. Generally speaking, most seizures terminate spontaneously. Closed-circuit video-EEG recordings have demonstrated that the majority of self-limiting seizures in fact last no longer than a few minutes (Theodore et al., 1994; Luders et al., 1993). As a result, the most recent definition by the ILAE Task Force on Classification and Terminology defined SE in humans as "a seizure that shows no clinical signs of arresting after a duration encompassing the great majority of seizures of that type in most patients or recurrent seizures without interictal resumption of baseline CNS function." Therefore, failure to recover consciousness between convulsions and persistence of a neurological deficit are considered integral to the diagnosis. Improved level of consciousness would be expected within 20 to 30 minutes of a convulsive seizure.

## **1.3.2** Electrical models of temporal lobe epilepsy

## 1.3.2.1 Kindling

Electrical kindling involves the repeated application of short electrical stimuli to limbic brain regions (hippocampus or amygdala) which produces a progressive increase in electrographic and behavioural seizures (Loscher, 2002). The clinical phenomenology produced is very similar to the human condition (Sato et al., 1990; Loscher, 1999) with neuropathological changes reminiscent of mesiotemporal sclerosis found in many patients with TLE (Loscher, 1999; Goodman, 1998). It is commonly used as a model of elicited, not spontaneous, seizures. The associated epiphenomena include increased seizure severity and duration, decreased focal seizure threshold, development of spontaneous seizures upon further stimulation and neuronal degeneration in limbic brain regions. Kindling is certainly a chronic model of epilepsy which offers the advantage that seizures can also be elicited at will (Loscher, 1999).

## 1.3.2.2 Self-sustaining limbic status epilepticus

Sustained electrical stimulation of the hippocampus or amygdala can be used to induce a period of SE characterized by recurrent focal and generalized seizures which are uninterrupted. This is followed by the development of neuropathological features consistent with human mesiotemporal sclerosis and the development of recurrent spontaneous seizures arising typically after 3-4 weeks (Loscher, 2002)

## 1.3.3 Chemoconvulsant models of temporal lobe epilepsy

#### 1.3.3.1 Kainic Acid

The neurotoxin kainic acid (KA) is widely used in rodentsto induce epileptogenesis (Dudek FE, 2006). It can be administered systemically, through intraperitoneal,
subcutaneous or intravenous injection, or can be focally injected into the hippocampus or infused intracerebroventricularly. The method of administration affects the resultant pattern of cell loss and damage. Restricted injection of KA into the hippocampus limits neuronal damage primarily to the CA3 region (Jarrard, 2002).

KA is a potent agonist of the AMPA/kainate class of glutamate receptors. The neurotoxic potency of KA is thirty–fold that of the excitatory neurotransmitter glutamate (Johnston et al., 1974). KA receptors are highly expressed in the hippocampus both presynaptically and postsynaptically (Bloss and Hunter, 2010) in addition to throughout the amygadala (Rogawski et al., 2003), basal ganglia (Jin and Smith, 2011), cerebellum (Wisden and Seeburg, 1993) and entorhinal cortex (Patel et al., 1986). Activation of KA receptors triggers membrane depolarization and excessive intracellular calcium influx. This in turn leads to neuronal death resulting from mitochondrial dysfunction and generation of reactive oxygen species (Nicholls, 2004; Schinder et al., 1996; Brorson et al., 1994). The KA model of TLE was initially developed by Ben-Ari and colleagues using an intra-amygdaloid injection of KA to induce the neuropathological features associated with human TLE (Ben-Ari and Lagowska, 1978; Ben-Ari et al., 1979).

#### 1.3.3.2 Pilocarpine

The pilocarpine-SE model displays many of the clinical and histopathological manifestations of human mTLE (Covolan and Mello, 2000; Bankstahl and Loscher, 2008; Turski et al., 1987b; Leite et al., 1990). The pilocarpine model of epilepsy in rats is probably the most extensively described pre-clinical model of epileptogenesis (Turski et al., 1987b; Turski et al., 1983). Although previously less well utilised in mice due to technical challenges, recently the model has been rigorously evaluated and optimized for use in drug discovery programmes (Mazzuferi et al., 2012). Systemic administration of pilocarpine induces a period of status epilepticus in rodents, characterized by generalised tonic-clonic convulsions. A latent period of variable duration follows with the appearance of spontaneous recurrent seizures (Leite et al., 1990; Cavalheiro et al., 1991) (chronic epilepsy). Reorganization of hippocampal tissue results, with characteristic mossy fibre sprouting, interneuron loss and ectopic dentate granule cell proliferation, features shared by human mTLE (Wieser, 2004). What is more,

pilocarpine-treated rodents are relatively unresponsive to AEDs (Glien et al., 2002; Chakir et al., 2006). Pilocarpine exerts its effect via the M1 muscarinic receptor subtype, causing an imbalance between excitatory and inhibitory transmission and an elevation in glutamate levels. Seizures are then maintained by N-methyl-D-aspartate (NMDA) receptor activation (Nagao et al., 1996; Smolders et al., 1997).

#### 1.3.3.3 Bicuculline

Bicuculline is an antagonist of the GABAA receptor (Curtis et al., 1970) which when locally applied causes epileptogenesis in the very early stages of postnatal development in rats (Soukupova et al., 1993). In contrast to the excitotoxic cell damage induced by KA (Balosso et al., 2008), bicuculline provokes seizures in the absence of neurodegeneration (Vezzani et al., 2000).

### **1.3.4** Behavioural manifestations of chemoconvulsant-induced seizures and Status Epilepticus

KA or pilocarpine (at variable doses depending on administration site and species) induces a period of repeated seizure activity. The chemoconvulsant is administered systemically or microinjected focally (e.g. into the hippocampus) or intraventricularly at doses sufficient to induce a seizure. This then induces a period of self-sustained status epilepticus (SSSE). The major limitation to models using systemic chemoconvulsants is high mortality. Most groups therefore limit the duration of SSSE to 1.5-2 hours by injection of the anticonvulsant diazepam. In both electrical and chemical models the duration of SSSE is crucial for the development of subsequent epilepsy and spontaneous epileptic seizures. It has been shown that if SSSE is interrupted at 30 minutes, spontaneous seizures do not arise (Lemos and Cavalheiro, 1995). Therefore, standard practice is generally to interrupt the SSSE after 90–120 minutes which allows reduced mortality but also a sufficient duration of SSSE to induce development of epilepsy (Loscher, 2002).

Elicited kindled seizures and spontaneous seizures arising following the latent period in post-SE models are very similar. Therefore, the severity of these seizures is commonly rated by a scale that has been developed for kindled seizures. The spontaneous seizures are either partial ('limbic') or secondarily generalized ('motor'), and are accompanied by paroxysmal discharges in the electroencephalogram (EEG) (Loscher, 2002).

#### 1.3.5 Acute seizure models

The maximal electroshock seizure (MES) test has been used for decades for screening anticonvulsant therapies and remains one of the gold standards in early stage AED testing (Rogawski, 2006). It involves non-epileptic animals which are induced to have a seizure by an electrical impulse. The test involves a stimulus of sufficient intensity to induce maximal tonic extension of the hind limbs (Castel-Branco et al., 2009). The stimulus is approximately 5-10 times higher than the individual seizures threshold of the animals to avoid the bias of daily fluctuations in seizure threshold (Loscher et al., 1991; Piredda et al., 1985; Swinyard and Kupferberg, 1985). The test is well standardized, has fixed parameters (in mice, 50 mA fixed current) and requires minimal expertise, as compared to the more intensive models involving SE. The stimulus is applied through transcorneal or transauricular (ear-clip) electrodes. In brief, the stimulus is applied followed by an immediate severe tonic seizure with maximal extension of the anterior and posterior legs and body stiffening. This is the tonic phase, usually lasting 10-15 seconds. After that, clonic seizures commence, characterized by paddling movements of the hind limbs and body shaking. The animal usually returns to an upright position within 20-30 seconds and starts moving around, apparently recovering its normal behaviour (Andre et al., 2002). The test is deemed positive if the animal exhibits tonic extensor seizure with rearward hind limb extension more than 90° from the body which is sustained for more than 3 seconds following 10 seconds after stimulation (Castel-Branco et al., 2009). Alternatively, acute seizures can be induced chemically. The subcutaneous pentylenetetrazol (PTZ)-induced seizure involves myoclonic jerking, clonic convulsion and/or tonic hindlimb extension. PTZ is a GABA-A receptor antagonist. Additionally, flurothyl is a convulsant fume that can induce a violent tonic clonic seizure in rodents, depending on their age (Sperber and Moshe, 1988). The mechanism of action is uncertain however increased sodium channel opening has been suggested (Woodbury, 1980).

#### **1.4** Epilepsy and Inflammation

Increasing evidence supports a link between inflammation and epilepsy, both in terms of epileptogenesis and the long term consequences of seizures (Vezzani et al., 2011a). Complex febrile seizures in childhood have long been associated with the later development of temporal lobe epilepsy, febrile illnesses in people with otherwise wellcontrolled epilepsy can trigger seizures, and immunomodulatory agents such as steroids and adrenocorticotrophic hormone have shown efficacy in some epileptic encephalopathies and occasionally in refractory status epilepticus (Hart et al., 1994; Snead, 2001). More recently, it has been reported that surgically resected brain tissue from individuals with refractory focal epilepsy displays all of the hallmarks of a chronic inflammatory state, with infiltration of leukocytes, reactive gliosis, and over-expression of cytokines and their target proteins (Vezzani et al., 2011a). This is supported by data from studies of animal models that confirm the intimate involvement of inflammatory mechanisms in the generation of epileptic discharges and in the cellular damage associated with focal-onset seizures (Vezzani and Ruegg, 2011). Targeting brain inflammation may accordingly represent a novel therapeutic strategy for epilepsy, consistent with efforts to shift the focus away from the symptomatic control of seizures to disease-modifying treatments that better target the underlying pathological mechanisms.

#### 1.4.1 The Immune System

The host adaptive defence against infection and tissue injury triggered by noxious stimuli can be divided into two general types of reactions: innate and adaptive immune reactions (Kumar, 2007; Nathan, 2002). As the name suggests, the innate immune system comprises cells that are ever present in order to respond to insults. These cells serve two main purposes: to trigger an immediate and contained inflammatory reaction to limit the spread of infection/injury and to trigger the second branch of immune reaction, adaptive immunity.

Response to microbial infection is the most well characterized innate immune system response and is triggered mainly by Toll-like receptors (TLRs) and nucleotide-binding oligomerization-domain protein-like receptors (NLRs) (Barton, 2008). These innate immune receptors are evolutionarily adapted to recognise specific, highly conserved

features of microbes; a process termed 'pattern recognition'(Janeway, 1989). The lipopolysaccharide (LPS) component of the gram negative bacterial cell wall is a wellrecognised example of such a pathogen-associated molecular pattern (PAMP). PAMPs are carried by all organisms of a certain class and the host has evolved a set of pattern recognition receptors (PRRs) able to detect their presence. In addition to pathogen recognition, the immune system also responds to so called 'sterile-injury', molecules that are released from damaged and dying cells, termed damage-associated molecular patterns (DAMPS) (Chen and Nunez, 2010).

In the brain, innate immunity is predominantly conferred by microglial cells, which act as the resident macrophages of the nervous system and represent the first line of defence against injury (Becher et al., 2000), but emerging evidence suggests that both neurons and astrocytes also play an important role (Vezzani et al., 2011d). Cytokines and chemokines released from activated microglia initiate a pro-inflammatory signalling cascade that ultimately leads to localised vasodilation, the extravasation and recruitment of leukocytes, and activation of the adaptive immune response, in which microglia also play a role by acting as antigen-presenting cells (Aloisi et al., 2000). Ordinarily, this process is halted by removal or elimination of the injurious stimulus, at which stage the immune response is scaled back and astrocytes and microglia turn their attention to repair, through the release of anti-inflammatory cytokines, the pruning of damaged synapses and the promotion of neuronal re-growth (Stoll et al., 2000). However, under circumstances that remain poorly understood, the resolution of inflammation is compromised, the proliferation of activated microglia is perpetuated and their attendant cytotoxic functions exaggerated. In chronic neuroinflammation, astrocytes and microglial cells appear to act in a deleterious manner, contributing to rather than reversing the neuronal damage, by the sustained release of pro-inflammatory cytokines and chemokines and proteases such as cathepsins and metalloproteinases (PL, 2003).

In addition to their role in immunological surveillance, microglia also play a crucial role in the healthy brain by monitoring and maintaining synapses (Nimmerjahn et al., 2005; Davalos et al., 2005). Synaptic pruning describes the process by which synapses are engulfed. Immunohistochemistry against the excitatory postsynaptic density marker PSD95 has shown that microglia engulf synaptic material. Knock-out mice for the chemokine fraktaline Cx3cl1, expressed exclusively by microglia (Jung et al., 2000; Harrison et al., 1998) and essential for their migration (Ruitenberg et al., 2008), are deficient at synaptic pruning (Paolicelli et al., 2011).

#### 1.4.1.1 Toll-like Receptors

TLRs are a class of at least 10 different protein receptors that span the cell membrane and contain both an extracellular, leucine-rich repeat (LRR) domain and an intracellular Toll/IL-1 domain (figure 1.3). TLRs link the recognition of extracellular PAMPs on bacteria, viruses, fungi and protozoa to the activation of their recognising cells which include dendritic cells (DCs), macrophages and neutrophils (Medzhitov, 2001; Akira et al., 2001). DCs act as the messenger between the innate and adaptive immune response, by presenting antigens to T-cells for recognition. Activation of TLRs initiates a downstream sequence of events leading to activated B cells (NF-κB) which in turn regulates the expression of genes that encode the inflammatory cytokines (Kawai and Akira, 2007).



*Figure 1.3 Toll-like receptors and some of their important ligands, adapted from (Kaufmann, 2007)* 

#### 1.4.1.2 Nod-like Receptors

In contrast to the ancient TLRs, the nod-like receptor (NLR) signalling mechanisms are less well characterised. NLRs are located in the cytosol and recognise intracellular DAMPS and PAMPs on pathogens that are able to penetrate the host cell (Ting et al., 2006; Inohara et al., 2005). NLRs comprise a large family (figure 1.4), the functions of which are diverse and determined by a variable amino-terminal domain. In addition they contain an LRR domain and a conserved NACHT domain. The Nod subfamily including Nod-1 and Nod-2 function similarly to TLRs via NF-kB and can in fact be synergistic; their activation can lead to enhanced cytokine production in certain situations (Kobayashi et al., 2005). The NLRP subfamily also contains an N-terminus pyrin domain (PYD). The NLRP gene family encodes the NACHT-, LRR-, and pyrin domain–containing proteins (Nalps) which control assembly and activation of the inflammasome (Mariathasan and Monack, 2007).



Figure 1.4 The domain structure of Nods in humans. The amino-terminal effectorbinding domains (EBDs) are variable in Nods, along with a variable number of leucinerich repeats (LRRs). Nods are composed of an EBD, a centrally located nucleotidebinding oligomerization domain (NOD) that mediates self-oligomerization, and a carboxyl-terminal ligand-recognition domain (LRD). Other abbreviations include  $\alpha$ ,  $\alpha$ helix/coiled-coil rich; NC, NALP1/CARDINAL expanded homology domain; WD40R, WD40 repeat; BIR, baculoviral inhibitor-of-apoptosis repeat. Figure adapted from (Inohara et al., 2005).

#### 1.4.1.3 Inflammasomes

Inflammasome complexes comprise three main components. They have a cytosolic PRR which is either an NLR or from the pyrin and HIN domain-containing (PHYIN) family of proteins. They also contain the procaspase-1 enzyme and an adaptor protein which enables interaction between the enzyme and receptor. Activation of NLRPs triggers recruitment of the adaptor apoptosis-associated speck-like protein containing a CARD (ASC) (Fernandes-Alnemri et al., 2009). ASC acts as the adaptor protein within the inflammasome between the PRR and the CARD of pro-caspase-1 (Case, 2011; Case and Roy, 2011; Faustin et al., 2007). Inflammasome assembly ultimately leads to cleavage of procaspase-1 to the active caspase-1, the enzyme that cleaves inactive precursor cytokines interleukin-1 $\beta$  (IL-1 $\beta$ ) and interleukin-18 (IL-18) into active moieties (Cerretti et al., 1992; Thornberry et al., 1992; Schroder and Tschopp, 2010). There are several known inflammasome complexes that form in response to an infectious or sterile injurious insult. The type and combination of the components

determines the inflammasome formed. All are essential for the activation of proinflammatory caspases necessary to initiate a potent inflammatory response.

The inflammasome that is activated by DAMPs under sterile conditions (such as may occur during epileptogenesis) is the NACHT, LRR and PYD-containing protein 3 (NLRP3) inflammasome. This comprises the PRR NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1 (figure 1.5).



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Figure 1.5 The Nod-like receptor, pyrin domain-containing 3 (NLRP3) inflammasome. Under normal conditions, NLRP3 is auto-repressed resulting from internal interaction between the NACHT domain and leucine-rich repeats. Upon exposure to pathogenassociated molecular patterns (PAMPs) from microorganisms or damage-associated molecular patterns (DAMPs) from endogenous danger signals, the NACHT domain is exposed and auto-repression ceases. As a result, NLRP3 oligomerizes and recruits apoptosis-associated speck-like protein containing a CARD (ASC; also known as PYCARD) and pro-caspase 1, triggering the activation of caspase 1 and the maturation and secretion of pro-inflammatory cytokines such as interleukin-16 (IL-16) and IL-18. CARD, caspase-recruitment domain; LRRs, leucine-rich repeats; NACHT, NAIP, CIITA, HET-E and TP1; PYD, pyrin domain. Figure reproduced with permission from (Tschopp and Schroder, 2010)

In the brain, the NLRP3 inflammasome is primarily expressed in microglia although evidence for functional NLRP3 has been shown in neurons (Walsh et al., 2014). The NLRP3 inflammasome has recently also been shown to exert pro-inflammatory, yet IL- 1-independent, effects including regulating the release of High Mobility Group Box-1 (HMGB1) (Lamkanfi et al., 2010).

#### 1.4.2 Cytokines

Cytokines are a diverse group of proteins produced by almost every cell and are involved in inter-cell communication of both the innate and adaptive immune response. They act to intensify the immunological response; binding to specific cellsurface receptors to induce intracellular signal cascades that alter cell function by up/down-regulation of genes and their respective transcription factors.

Generalization of cytokine function is difficult as their action is dependent upon a complex series of interactions between the cytokine and specific receptor, the role of the receptor being of equal importance. It is possible, however, to classify function into those that are broadly pro-inflammatory and those that are anti-inflammatory according to the up-regulation of genes coding for synthesis of mediator molecules during inflammation, namely type II phospholipase (PL) A2, cyclooxygenase (COX)-2, and inducible nitric oxide (NO) synthase. The enzymes encoded by these genes increase the synthesis of platelet-activating factor and leukotrienes, prostanoids, and NO. Pro-inflammatory cytokines include mainly the interleukin (IL)-1 family, IL-2, IL-6, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and interferon-y (IFNy). Cytokine gene expression is triggered by various cell stressors including infection, inflammatory products, ultraviolet light, heat-shock and hyperosmolarity. All of these activate the mitogenactivated protein kinases (MAPKs), which phosphorylate transcription factors for gene expression (Dinarello, 2000). The stimulus for the synthesis of pro-inflammatory genetic cascades is primarily the synergistic action of IL-1 and TNFα. IL-1 and TNF are potent inducers of endothelial adhesion molecules which are essential for recruitment of leukocytes into tissue via adhesion to the endothelial surface (Dinarello, 2000). Conversely, anti-inflammatory cytokines suppress the intensity of this cascade and include IL-4, IL-10, and IL-13. They have the ability to suppress genes for IL-1, TNFα and the chemokines. It must be borne in mind, however, that many cytokines have pleiotropic activity and may function differently depending upon the biological circumstances. For example, IFNy is considered a pro-inflammatory cytokine because it augments TNF activity and induces NO. However it also possesses antiviral activity and can activate cytotoxic T-cells. It is likely that an effective balance between the effects of pro-inflammatory and anti-inflammatory cytokines determines the outcome of disease.

#### **1.4.2.1** Inflammatory mediators and epilepsy

Recent studies point to a significant contribution of inflammation in the pathophysiology of symptomatic epilepsy (table 1.6).

#### 1.4.2.2 Interleukin-16 and epilepsy

Interleukin-1 $\beta$  (IL-1 $\beta$ ) is probably the most extensively studied and important proinflammatory mediator. Much of the early evidence to support a role for inflammation in epilepsy arose from studies of IL-1 $\beta$ , its target, interleukin-1 receptor type 1 (IL-1R1), and its naturally occurring competitive antagonist, interleukin-1 $\beta$  receptor antagonist (IL-1RA) (Vezzani et al., 2000; Viviani et al., 2003; Vezzani et al., 2011d). All three are up-regulated in rodent brain as a result of seizures in a variety of experimental models, including those induced by electrical stimulation, kainate, bicuculline, and hyperthermia (Eriksson et al., 1999; Viviani et al., 2003; Dube et al., 2005; Ravizza and Vezzani, 2006). IL-1 $\beta$  expression in glial cells has been shown to remain elevated for up to 60 days after experimental status epilepticus (De Simoni et al., 2000) and elevated IL-1 $\beta$  has been reported in CSF from children with febrile seizures (Haspolat et al., 2002; Ravizza et al., 2008h). Elevated IL-1 $\beta$  and IL-1R1 have also been observed in brain tissue from patients with a variety of pathologies including medial temporal lobe epilepsy with hippocampal sclerosis, focal cortical dysplasia, and tuberous sclerosis (Boer et al., 2008; Ravizza et al., 2006a; Ravizza et al., 2008a; Crespel et al., 2002). Similarly, several inflammasome-associated genes (IL-1β, IL-18, NLRP1, NLRP3, and caspase-1) showed increased transcript levels in brain tissue from patients with the epileptic encephalopathy Rasmussen's encephalitis (RE) as compared to non-RE controls (Ramaswamy et al., 2013). In addition to their over-expression arising as a result of seizures, IL-1 $\beta$  and IL-1RA can also modulate susceptibility to seizure-inducing stimuli. When injected directly into the CNS, IL-1 $\beta$  exacerbates seizures induced by Table 1.6 Studies examining the contribution of inflammatory mediators to epilepsy in experimental models and human epilepsy.

Inflammatory mediators	Population	Outcome	Comments	Ref
Interleukin-1β	Rat model of epilepsy (Induced by KA and biccuculine)	Both KA and bicuculine-induced seizures cause rapid increase in hippocampal IL-1β expression. Intrahippocampal injection of IL-1β 10 min before KA enhanced by 226% the time spent in seizures	The proconvulsant effect was blocked by co-administration of the antagonist, IL-1RA.	(Vezzani et al. <i>,</i> 1999)
IL-1 $\beta$ , IL-6, and TNF $\alpha$	Rat limbic status epilepticus model	Induction of IL-1β, IL-6, TNFα and inducible nitric oxide 6-24 hours after seizures. Intracerebral injection of IL-1RA significantly reduces behavioural seizures.		(De Simoni et al., 2000)
IL-1β and IL-1RA gene polymorphisms	Human focal epilepsy genomic DNA	Strong association between homozygotes for the pro- inflammatory IL-1β polymorphism (-599) in patients with TLE and HS as compared to non-epileptic controls and those with TLE without HS.	Suggests a subtle anomaly during development could lead to HS in genetically susceptible individuals.	(Kanemoto et al., 2000)
IL-1β, IL-6 and TNFα	Rodent limbic seizure model (induced by KA, biccuculine or electrical)	Limbic seizures rapidly and transiently enhanced IL-1β, IL-6 and TNFα mRNA in the hippocampus. IL-1β remained persistently elevated 60days following seizure. Transgenic mice overexpressing ILI- RA showed decreased susceptibility	Pre-Injection of IL-1β enhanced the time spent in seizures; the effect was blocked by IL-1RA.	(Vezzani et al., 2002)

		to bicuculline induced seizure		
ΙL-1β	Rat hippocampal neuron cultures	IL-1β dose-dependently enhanced NMDA-induced calcium increases with phosphorylation of the NR2A/B subunit which mediates excitotoxic neuronal death.	The effect on the NMDA subunits was abolished by co- administration of IL-1RA	(Viviani et al. <i>,</i> 2003)
IL-1β, IL-1RA and IL-6	Human drug-resistant epilepsy (10) plasma	Highly pro-inflammatory cytokine profile (high IL-6, low IL-1Ra and low IL-1Ra/IL-1beta ratio) observed in plasma from patients with epilepsy.	IL-1Ra significantly lower in epilepsy patients compared to controls (p<0.001)	(Hulkkonen et al. <i>,</i> 2004)
Interleukin-1β family (IL- 1β, IL-1RI, IL-1RII, IL-1RA)	Human drug-resistant epilepsy, brain tissue from focal cortical dysplasia and glioneuronal tumors (27)	Moderate to strong expression IL- 1β and IL-1RI in all specimens. Neuronal staining was positively correlated with seizure frequency.	Developmental lesions associated with intractable epilepsy, high expression of IL-1β and its receptor and paucity of expression of natural antagonists (IL1-RII, IL-1RA) suggests ineffective inhibitory control.	(Ravizza et al., 2006a)
IL-1β, interleukin- converting enzyme (ICE/caspase-1)	Rat model of epilepsy (KA), Caspase-1 KO mice and Organotypic hippocampal slice cultures	Selective inhibition of ICE/caspase- 1 or caspase-1 gene deletion delayed time to seizure onset and number of seizures.	Selective inhibition of brain IL-1β represents a novel anti- convulsant strategy.	(Ravizza et al., 2006e)
IL-1β IL-1RI	Rat models of epilepsy (induced by pilocarpine and SSLSE) and	Neuronal and glial activation of IL- 1 $\beta$ system. Monocytes and macrophages detected in areas of neuronal loss. IL-1 $\beta$ and IL-1RI	Activation of IL-1 system during epileptogenesis may increase neuronal excitability and alter BBB permeability.	(Ravizza et al., 2008a)

	Human adult drug- resistant TLE (18) undergoing resection.	strongly immunoreactive in microvasculature in areas of BBB damage.		
TGF-β and BBB dysfunction	Rat focal injury model and hippocampal slice cultures.	Focal BBB breakdown activates a TGF-β receptor mediated signalling cascade in glia and local inflammation	Damage to the microvasculature leads to serum extravasation into the brain and activation of TGF- $\beta$ cascade.	(Cacheaux et al. <i>,</i> 2009)
Leukocyte adhesion molecules	Mouse epilepsy model pilocarpine	Seizure-induced up-regulation of vascular leukocyte adhesion molecules (I-CAM, VCAM-1, E- selection, P-selectin)	Antibody inhibition of leukocyte- vascular interactions markedly reduced seizures, suggests a pathological link between leukocyte-vascular interactions, BBB damage and seizure generation.	(Fabene et al., 2008)

IL-1R1: Interleukin-1 type-1 receptor; IL1-RII: Interleukin-1 type II receptor; IL-1RA: Interleukin-1 receptor antagonist; IL-6: Interleukin-6; TNFα: Tumour necrosis factor-α; mRNA: messenger-RNA; NMDA: N-methyl-D-aspartate receptor; KO: knock-out; TLE-HS: temporal lobe epilepsy with hippocampal sclerosis; KA: Kainic acid; RT-PCR: Reverse transcription-polymerase chain reaction); SSLSE: Self-sustained limbic status epilepticus; TLE: temporal lobe epilepsy; TGF-8: Transforming growth factor-8; BBB: Blood-brain barrier; ICAM-1: Intercellular Adhesion Molecule-1; VCAM-1: vascular cell adhesion molecule 1; E-selectin: endothelial; P-selectin: platelet; PBMC: peripheral blood mononuclear cell. Table reproduced from (Walker et al., 2015). kainic acid and bicuculline (Viviani et al., 2003) and lowers the seizure threshold in models of febrile convulsions (Dube et al., 2005; Heida and Pittman, 2005). In contrast, IL-1RA has anticonvulsant activity following intracerebral administration and transgenic mice that over-express this protein in astrocytes have reduced seizure susceptibility (Vezzani et al., 2000; Auvin et al., 2010b). Similarly, IL-1R1 knockout mice are less sensitive to experimentally-induced febrile seizures (Dube et al., 2005) and the convulsant effects of bicuculline (Vezzani et al., 2000).

The pro-convulsant effects of IL-1 $\beta$  are believed to be mediated via IL-1R1 dependent activation of neuronal sphingomyelinase and Src kinases, resulting in phosphorylation of the NR2B subunit of the NMDA receptor, stabilisation of the receptor at the cell surface, enhanced NMDA-mediated calcium (Ca<sup>2+</sup>) conductance, and an increase in glutamatergic neurotransmission and the propensity for excitotoxicity (Viviani et al., 2003; Balosso et al., 2008). Other putative effects of IL-1 $\beta$  include a reduction in astrocytic glutamate uptake (Hu et al., 2000), an enhanced release of glutamate from glial cells, possibly via enhanced TNF- $\alpha$  production (Bezzi et al., 2001), and the generation of acquired channelopathies (Viviani et al., 2007).

#### 1.4.3 Pyroptosis

Pyroptosis is a form of programmed cell death mediated by caspase-1. Pyroptosis is dependent on the NLRP3 inflammasome complex and its unique feature includes loss of membrane integrity accompanied by an outflow of intracellular inflammatory components, most notably high mobility group box-1 (HMGB1) (Scaffidi et al., 2002).

#### 1.5 Epilepsy and inflammation

#### **1.5.1** Disruption of the blood brain barrier.

The CNS was previously presumed to be an immunologically privileged site, separated from perturbations in the microenvironment of the peripheral blood by the mechanical barrier formed by the BBB. The resident astrocytes and microglia maintain independent CNS immunity. However, it is now understood that this concept of "privilege" is not wholly absolute and in some conditions, disruption of the BBB permits a link between the peripheral and central immune systems. Using two-photon microscopy to examine the dynamics of lymphocytic choriomeningtis virus (LCMV) infected mice, fluorescently labelled CD8+ T-cells have been visualised in the mouse brain after infection (Kim et al., 2009). Immediately following intracerebral inoculation, CD8+ T-cells entered the subarachnoid space and contact LCMV infected stromal cells. The resultant T-cell activation leads to chemokine excretion, influx of monocytes and neutrophils and production of inflammatory cytokines. This inflammatory recruitment process results in disruption of the BBB and, ultimately, the development of fatal seizures in the mice. Furthermore, depletion of both monocytes and leukocytes in the mice using anti-CD8+ antibody significantly preserved vascular integrity, prevented BBB leakage and prevented rapid onset of seizures 6 days after inoculation with LCMV (Kim et al., 2009).

Possible mechanisms for BBB breakdown in seizure disorders have been proposed. Upregulation of adhesion molecules, essential intermediaries for leukocyte recruitment into the CNS, has been identified in rodent models of seizures and epilepsy (Fabene et al., 2008). Peripheral injection of the chemoconvulsant pilocarpine is known to induce a mild inflammatory reaction with IL-1ß release (Marchi et al., 2007). Elevated expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), P-selectin and E-selectin was identified 1 and 7 days following pilocarpine-induced SE (Fabene et al., 2008). These adhesion molecules are a hallmark of tissue inflammation. Disruption of the leukocyte-endothelial cell interaction resulted in a marked reduction of spontaneous convulsions during the chronic period, with reduced seizure frequency. Furthermore, when an antibody to  $\alpha 4$  integrin was given 2 hours before pilocarpine, it completely prevented convulsions (Fabene et al., 2008). This model argues convincingly for a role of leukocyte-endothelial cell interaction and consequent BBB leakage in epileptogenesis. Changes in expression of several potassium and glutamate homeostasis related genes in response to BBB breakdown in rats have been identified (David et al., 2009). During BBB breakdown, serum-derived albumin diffuses into the extracellular space leading to rapid up-regulation of the astrocytic marker glial fibrillary acidic protein (GFAP), followed by delayed development of an epileptic focus (David et al., 2009; Seiffert et al., 2004). The TGF- $\beta$ signalling pathway is a candidate mechanism for delayed epileptogenesis following extravasation of serum albumin into the brain (Tomkins et al., 2007). In rats in whom the BBB has been disrupted with either deoxycholic acid, serum derived albumin or TGF-β1; gradual development of hypersynchronous neuronal epileptiform activity is seen. Co-immunoprecipitation revealed a direct interaction between albumin and the TGF-β type II receptor (RII). Following activation of this receptor, phosphorylation of intracellular proteins called SMADs occurs. Phosphorylated SMAD 2 and/or 3 form a complex with the common mediator protein SMAD 4, resulting in translocation to the nucleus and transcriptional activation (Pavlovsky et al., 2005). The pathophysiological cascade leading to pro-inflammatory activation in epilepsy is described in figure 1.6.

In humans, intravenous mannitol can be used to temporarily disrupt the BBB to allow intrathecal delivery of chemotherapy for the treatment of brain lymphoma. In 8 patients undergoing BBB disruption for this purpose, 25% of 102 procedures resulted in motor seizures (Marchi et al., 2007). There were no seizures in patients receiving chemotherapy without BBB disruption. Seizures occurred exclusively within the time frame of the procedure and despite heavy premedication with the anticonvulsant thiopental. Findings were subsequently confirmed in an animal model, removing the potential confounders of chemotherapy and presence of lymphoma.

However, BBB disruption is not necessarily a prerequisite of seizure activity, demonstrated elegantly *in vitro* in the isolated guinea pig brain (Librizzi et al., 2012). In this model seizures induced by the convulsant drug bicuculline have been shown to induce production of inflammatory mediators, despite the complete absence of blood-borne molecules (Librizzi et al., 2012). This model demonstrates clearly that seizure activity alone is sufficient to induce brain-borne inflammation, in particular IL-1 $\beta$ . Targeting biologically active IL-1 $\beta$  using the human recombinant interleukin-1 receptor-1 antagonist anakinra, reduced seizure duration by 80% in this model. In addition, seizures in this model induced long-standing BBB breakdown.

Taken together, substantial evidence supports a temporal and what is more, causal relationship between BBB disruption, inflammatory activation and seizure generation.

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#### 1.5.2 Autoimmunity

#### 1.5.2.1 Auto-antibodies

Autoimmune activation has been associated with certain epilepsy syndromes (Peltola et al., 2000a; Eriksson et al., 2001; Ranua et al., 2004; Rogers et al., 1994; Bartolomei et al., 1996), further suggesting that immune mechanisms are integral to seizure disorders. Numerous autoantibodies have been linked to seizure disorders including those targeted against voltage-gated potassium channels (VGKCs), voltage-gated calcium channel (VGCCs), Glutamate Receptor (GluR)3, glutamic acid decarboxylase (GAD), and the NMDA-subtype of glutamate receptor (NMDA-R).

Antibodies against (GluR)3 (and GluR2 at low concentrations) have been detected in the serum of some patients with Rasmussen's encephalitis (Rogers et al., 1994). Experimental evidence suggests that these antibodies may activate cortical neurons and induce complement dependent and independent cytotoxicity (Twyman et al., 1995; He et al., 1998; Levite and Hermelin, 1999), but, confirmatory clinical studies determining the frequency of these antibodies in consecutive series of patients, or in the general population are absent. Limbic encephalitis (LE) predominantly affects the medial temporal lobes characterised by seizures, psychiatric disturbance and loss of short-term memory. It can be both paraneoplastic, in association with certain tumour types (Darnell and Posner, 2003), or non-neoplastic in origin, wherein it is associated with elevated VGKC and NMDA receptor antibodies (Majoie et al., 2006; Reid et al., 2009). GAD antibodies have also been reported in patients with stiff person syndrome (SPS) (Levy et al., 2005) and more recently, in patients with temporal lobe epilepsy (Liimatainen et al., 2010). Theoretically, inhibition or loss of GAD caused by these antibodies could cause a reduction in GABA synthesis with resultant reduced neuroinhibitory activity and lowered seizure threshold. In vitro studies support a pathogenic role in the development of epilepsy.



Figure 1.6 Schematic detailing the pro-inflammatory pathways involved in epilepsy. Events including neuro infection, head injury, prolonged febrile seizure result in activation of microglia, astroctyes and neurons and disruption of the blood brain barrier (BBB). Pro-inflammatory cytokines and damage associated molecular patterns (DAMPS) including, IL-16 and high mobility group box-1 (HMGB1), are released into the extracellular milieu. Signalling activation in neurons results in a rapid increase of NMDA receptor calcium (Ca<sup>2+</sup>) conductance via Src mediated phosphorylation of the NR2B subunit. This in turn leads to increased intracellular Ca<sup>2+</sup>, which causes neuronal hyperexcitability, decreased seizure threshold and network reorganization. Activation of the NFkB-dependant transcription of genes contributes to molecular and cellular changes involved in epileptogenesis, and perpetuates brain inflammation. The initial inciting events described may also cause glutamate-mediated activation of NMDA receptors in neurons which in turn promotes COX-2 activation via inducible Nitric Oxide (iNOS) and Phospholipase A2-mediated production of arachidonic acid (AA). Production of prostaglandin E2 activates EP1 and EP2 receptors which are coupled to intracellular signalling involving Ca<sup>2+</sup> mobilization from intracellular stores and cAMP production. As a consequence of BBB disruption, serum-derived albumin passes into brain parenchyma, causing TGF-B signalling in astrocytes leading to NFkB dependent gene transcription, astrocyte dysfunction (decreased Kir4.1 and glutamate transporter) and pathological outcomes. Neuronal network hyperexcitability, cell injury, and network reorganization results, which are then responsible for the onset of seizures and the development of epilepsy. Figure adapted from (Vezzani et al., 2013).

Strong immunoreactivity is observed on application of GAD-Ab-positive sera from epilepsy patients to hippocampal neurons in culture. No reactivity was observed with sera from antibody negative patients, healthy control individuals or GAD-Ab positive type-1 diabetic patients (Vianello et al., 2006). Whether anti-GAD Abs are directly responsible for reduced activity of GAD in GABAergic neurons and hence reduced GABA levels or whether they are epiphenomena, produced secondary to damage to GABAergic neurons, remains unclear at present. Co-existent intrathecal synthesis (Liimatainen et al., 2010) would suggest an ongoing immune response and highlights those patients that may benefit from immunomodulatory therapy.

#### 1.5.2.2 Immunomodulatory Therapies

Immunomodulatory interventions have proven efficacy in some of the catastrophic epilepsy syndromes of childhood which are otherwise resistant to antiepileptic drugs. Corticosteroids, intravenous immunoglobulins (IVIg) or plasmapheresis have been reported to be beneficial for Rasmussen's encephalitis (Hart et al., 1994; Andrews et al., 1996), although no blinded, placebo-controlled trials have been undertaken to confirm efficacy. Likewise, numerous un-blinded studies have shown IVIg to be efficacious in the treatment of infantile spasms and Lennox-Gastaut syndrome (van Engelen et al., 1994a; van Engelen et al., 1994b; van Engelen et al., 1994c; Echenne et al., 1991; Duse et al., 1996), despite their differing clinical phenotypes.

#### **1.5.2.3** Autoimmune disorders and epilepsy

The incidence of seizure disorders in systemic lupus erythematosus (SLE) is greater than that of the general population at 5.4%-10% (Mackworth-Young and Hughes, 1985; Formiga et al., 1997; Liou et al., 1996; Herranz et al., 1994). SLE is a complex, multisystem disorder, predominantly affecting women during reproductive years, in which the CNS is commonly affected and autoantibody production, including antinuclear, anticardiolipin and anti-double-stranded DNA antibodies, are a frequent feature. Many theories exist exploring the relationship between autoantibodies and seizures in SLE, including anti-brain antibodies directly causing seizures (Rogers et al., 1994; Mihailovic and Cupic, 1971), inhibition of chloride currents through the GABA receptor complex (Liou et al., 1994) and antiphospholipid antibodies directly affecting CNS tissue resulting in microvascular lesions (Liou et al., 1996). Given the growing body of evidence, it seems likely that serum autoantibodies may be associated with some forms of epilepsy; however, it remains unclear whether they arise as a cause or a consequence of seizures.

#### 1.6 The High-Mobility Group Superfamily

The high mobility group (HMG) superfamily members are responsible for mediating most of the structural changes in chromatin, a dynamic supramolecular nucleoprotein (Kornberg and Lorch, 1999). Genomic DNA in eukaryotic cells must be tightly compacted in the form of chromatin in order to fit into the cell nucleus. Access of regulatory factors to their cognate DNA binding sites on chromatin is achieved by folding and remodelling of the chromatin structure. Loosening this structure or even disruption of the nucleosome structure (by chromatin remodelling complexes), is achieved by DNA bending and unwinding, as well as by affecting the strength of DNA– histone interactions by DNA methylation, post-translational modifications of histones, or incorporation of specific histone variants to chromatin. This allows access of specific transcription factors or other proteins (Venters and Pugh, 2009). The HMG proteins have been subdivided into three distinct structural families: HMGA (the HMG-AT-hook family), HMGN (the HMG-nucleosome binding family), and HMGB (the HMG-box family) (Bustin, 2001b; Gerlitz et al., 2009).

#### **1.6.1** High Mobility Group A (HMGA) family

The mammalian HMGA protein family currently consists of four functionally active members, HMGA1a, b and c (alternative splice variants of one gene) and HMGA2 (Nagpal et al., 1999). HMGA proteins are architectural transcription factors that both positively and negatively regulate the transcription of a variety of genes (Cleynen and Van de Ven, 2008). The DNA-binding regions of the HMGA proteins assume a planar, crescent-shaped configuration called the 'AT-hook' when specifically bound to the minor groove of short stretches of AT-rich DNA (Reeves and Nissen, 1990). HMGA proteins recognise DNA structure and are able to bend, straighten, unwind and induce looping in linear DNA molecules in order to regulate gene expression (Reeves, 2001; Evans et al., 1995)

#### **1.6.2** The High Mobility Group nucleosome binding (HMGN) family

The High-mobility group nucleosome binding (HMGN) family contains 5 members, HMGN1-5. The hallmark of the family is the highly conserved nucleosomal binding domain (Bustin, 2001a; Ueda et al., 2008; Crippa et al., 1992). All HMGN's bind specifically to the 147 bp nucleosome core particle which is the building block of the chromatin fiber (Ueda et al., 2008). In this way they alter the structure and activity of chromatin and reduce compaction (Kugler et al., 2012).

#### **1.6.3** High Mobility Group Box (HMGB) family

The High-Mobility Group Box (HMGB) family consists of 3 recognised members: HMGB1, -2 and -3 (Stros, 2010). The HMGB proteins all consist of two DNA binding domains (termed the A and B boxes) and a long acidic carboxyl terminus (Bianchi et al., 1989). HMGB1 is a 25KDa highly conserved protein discovered 30 years ago (Goodwin et al., 1973) that has 99% sequence homology between mammals. It is widely expressed in most eukaryotic cells in several animal species, humans among them (Yang et al., 2005a). This reflects distinct biological functions despite wide species variation. HMGB1 is ubiquitous, with lowest tissue levels found in brain and liver (Mosevitsky et al., 1989) and will be discussed in detail in this review.

#### 1.6.3.1 High Mobility Group Box 2 and 3

In brief, HMGB2 is highly expressed during embryogenesis and in adults, it is mainly expressed in testicles and lymphoid organs. Mice lacking the HMGB2 gene are viable, but knockout males have reduced fertility (Stros, 2010, Ronfani et al., 2001.) HMGB2 protein overexpression has been demonstrated in hepatocellular carcinoma (Kwon et al., 2010b) and glioblastoma multiforme, wherein it is also correlated with shorter overall survival time (Wu et al., 2013). It has also been shown to be down-regulated in colorectal cancer cell lines (Shin et al., 2013). HMGB3 has been mapped to the X chromosome band q28 (Gao et al., 2015). It plays an important role in regulating the balance between haematopoietic stem cell proliferation and differentiation (Nemeth et al., 2006). Overexpression of HMGB3 mRNA has been found in metastatic breast cancers (Elgamal et al., 2013), which correlates with poor survival. More recently, overexpression has been linked to poor prognosis in oesophageal squamous cell carcinoma (Gao et al., 2015).

#### **1.6.3.2** High Mobility Group Box-1

HMGB1 is a 25KDa highly conserved protein comprising three major protein domains; two DNA binding regions (termed the A and B boxes) and a 30 amino acid acidic carboxyl terminus (Bianchi et al., 1989). Discovered 30 years ago (Goodwin et al., 1973), it has myriad intracellular and extracellular functions.

#### 1.1.1.1.1 Nuclear role

Primarily nuclear, HMGB1 bends deoxyribonucleic acid (DNA) and regulates transcription. It functions as an architectural factor to support the structure of chromatin through interaction with a myriad proteins to promote formation of nucleoprotein structures (Stros, 2010). Increasing the flexibility of DNA can recruit binding of additional transcription factors, achieved by looping and bringing the factors into closer proximity (Stros et al., 1994; Becker et al., 2008; Paull and Johnson, 1995). In addition, ATP-driven chromatin remodelling, giving access of specific DNA sites to transcription factors is enhanced by HMGB1 (Bonaldi et al., 2002).

HMGB1 contains two nuclear localisation sequences (NLSs) located in the A box (NLS1, amino acids 28-44) and in the B box (NLS2, amino acids 179-185), respectively (Yang et al., 2013). Four conserved lysine residues are present in NLS1, and five are present in NLS2. They are susceptible to acetylation modification which signals migration to the cytoplasm where it is packaged into vesicles for release (Wang et al., 1999; Evankovich et al., 2010; Bell et al., 2006; Rovere-Querini et al., 2004; Kazama et al., 2008; Bonaldi et al., 2003; Scaffidi et al., 2002). Release occurs through a non-classical pathway as HMGB1 lacks the necessary leader signal sequence required for the classical endoplasmic-reticulum mediated pathway. In response to an inflammatory signal, acetylated HMGB1 is released following fusion of the vesicles with the plasma membrane (Nickel and Rabouille, 2009; Lamkanfi et al., 2010). HMGB1 can be released passively, from the nucleus of damaged or necrotic cells, or actively as part of the innate immune response from many different cells types including macrophages, monocytes, natural killer cells, endothelial cells, and platelets (Harris et al., 2012; Yang et al., 2013). Therefore depending upon the presence or absence of acetylation

modifications, it is possible to identify the mode of HMGB1 release (necrotic or immune-mediated).

#### 1.6.3.2.1 Acetylation modifications

Acetylation of HMGB1 near the nuclear localization sequences blocks communication with the nuclear importer and inhibits re-entry into the nucleus (Lotze and Tracey, 2005). Lysine residues susceptible to acetylation within the HMGB1 molecule act as markers of release mechanisms; non-acetylated HMGB1 is passively released during necrotic cell death, whereas acetylation promotes active release from immune cells following infection and sterile inflammation (Lamkanfi et al., 2010; Andersson et al., 2014)

#### 1.6.3.2.2 Cytokine role and redox modifications

HMGB1 contains three cysteine residues at positions 23, 45, and 106, which are sensitive to redox-dependent modifications. Once outside the cell, HMGB1 can act as a chemo-attractant through formation of a heterocomplex with the chemokine C-X-C motif chemokine 12 (CXCL12), together acting in synergy upon the CXC-receptor type 4 (CXCR4) receptor in order to recruit leukocytes (Venereau et al., 2013). To form this effective heterocomplex, HMGB1 must be in the fully reduced form whereby all three cysteine residues contain a thiol group. It is in this fully reduced form that it is strictly referred to as simply "HMGB1" and more fully, HMGB1C23hC45hC106h, indicating the presence of a thiol group at each cysteine (Antoine et al., 2014). Through binding to the Toll-like Receptor 4 (TLR4), HMGB1 stimulates cytokine release and inflammation. Successful cytokine activation requires both the presence of a thiol group at C106 (mandatory for HMGB1 to bind to the TLR4/MD2 complex (Schiraldi et al., 2012)) and a disulphide bond between C23 and C45 (Yang et al., 2012). This particular variant of HMGB1 is termed "disulphide HMGB1" or more specifically, HMGB1C23-C45C106h to indicate a single intramolecular disulphide bond between C23 and C45 and no posttranslational modification on C106 (Antoine et al., 2014). These specific redox modifications (figure 1.7) directly dictate the cytokine and chemotactic activities of HMGB1. In addition to binding via TLR4, extracellular HMGB1 also binds to the multiligand receptor for advanced glycation end products (RAGE). RAGE has various binding

partners, including HMGB1, that are released during cellular or physiological stress(Sims et al., 2010). The promoter contains multiple functional NF-κB and specificity protein-1\* transcription factor–binding sites (Li et al., 1998; Li and Schmidt, 1997) and therefore ligand binding can potentially trigger an auto-inflammatory loop. To date, less is known about the HMGB1/RAGE axis however recent studies have suggested a role in neutrophil recruitment towards necrotic tissue, in the absence of macrophage-mediated signaling (Huebener et al., 2015).

Once outside the cell, HMGB1 functions as a potent alarmin to activate innate and adaptive immunity. The absence of HMGB1 in mice is lethal; HMGB1-/- knockout pups die within 24 hours owing to hypoglycaemia (Calogero et al., 1999).

#### 1.6.3.2.3 Identification

Total HMGB1 levels in serum, plasma or CSF can be quantified using a commercially available enzyme-linked immunosorbent assay (ELISA). Currently there are no specific antibodies able to detect the different molecular forms of HMGB1. Liquid Chromatography-Tandem Mass Spectrometry (LCMS/MS) is the only analysis method able to identify the structural and functional modifications of HMGB1 (Antoine et al., 2012; Antoine et al., 2009).

#### 1.7 The role of HMGB1 in epilepsy

#### 1.7.1 HMGB1 expression

Following seizure, IL-1β and HMGB1 are primarily expressed in microglia, although HMGB1 is also found in neurons (Eriksson et al., 1999; Maroso et al., 2010). Increased immunostaining for HMGB1 and its receptors, TLR4 and RAGE, has been demonstrated in the brain tissue of mice following induction of both kainate- and bicuculline-induced acute seizures and kainate-induced chronic epilepsy (Maroso et al., 2010; Iori et al., 2013). Increased staining for HMGB1, TLR4 and RAGE, is detectable in an analogous pattern to that observed in mouse models of epilepsy in human hippocampal tissue obtained at surgery from patients with temporal lobe epilepsy and hippocampal sclerosis, and also in malformations of cortical development associated with

intractable seizures (Iori et al., 2013; Maroso et al., 2010; Maroso et al., 2011b; Zurolo et al., 2011).



Figure 1.7 Different isoforms of high mobility group Box-1 (HMGB1) resulting from post-translational modifications. Reduced HMGB1 contains thiol groups on all 3 conserved cysteine residues and functions as a chemoattractant. Disulphide HMGB1 contains a disulphide bond between cysteine 23 and 45 and a thiol at cysteine 106. Through binding to the toll-like receptor 4 it stimulates release of pro-inflammatory mediators and the inflammatory cascade. Sulfonyl HMGB1 is fully oxidised contains a sulfonyl group on each cysteine and is considered to date to be immunologically inert. All forms of HMGB1 contain lysine residues that are susceptible to acetylation modification. Acetylated HMGB1 originates from inflammatory cells and as a consequence of acetylation, can no longer re-enter the nucleus. Non-acetylated HMGB1 is nuclear and is released by damaged and dying cells following nuclear burst.

#### **1.7.2 HMGB1 and IL-1β exacerbate seizures**

Microglia and astrocytes produce multiple inflammatory mediators in response to HMGB1 stimulation (Andersson et al., 2008; Pedrazzi et al., 2007). What is more, IL-1 $\beta$  can induce release of HMGB1 in both human (Zurolo et al., 2011) and rat (Hayakawa et al., 2010) cultured astrocytes. This suggests the possibility of a self-perpetuating feedback loop driven by both HMGB1 and IL-1 $\beta$ .

In rodents, pre-treatment with intra-hippocampal HMGB1 and IL-β prior to treatment with bicuculline or kainate exacerbates seizures (Vezzani et al., 1999; Maroso et al., 2011b; Vezzani et al., 2002). In contrast, intra-cerebral infusion of the endogenous IL-1R1 antagonist IL-1Ra or its over-expression in astrocytes delays seizure onset and reduces duration following kainate (Vezzani et al., 2000) or reduces seizure behaviour

following bicuculline treatment or electrically-induced SE (Vezzani et al., 2002). Seizure onset is also delayed in mice lacking IL-1R1 (Vezzani et al., 2000). Similarly, selective inhibition of HMGB1 or TLR4 delays seizure onset and decreases seizure number and duration in both kainate- and bicuculline-induced acute seizure models and reduces the number of spontaneous epileptic seizures in the kainate model of chronic epilepsy (Maroso et al., 2011b). Knock-out of TLR4 or RAGE is also anticonvulsant in kainate models of acute and chronic seizures (Iori et al., 2013). Increased expression of IL-1β and HMGB1 signalling in a variety of experimental models and seizure disorders (Maroso et al., 2010; Maroso et al., 2011b; Zurolo et al., 2011; De Simoni et al., 2000; Plata-Salaman et al., 2000), in addition to their established pro-convulsive effects (Maroso et al., 2010; Vezzani et al., 1999; Sayyah et al., 2005), provides evidence that targeting IL-1β and HMGB1 may prove successful in the treatment of epilepsy.

#### 1.7.3 Pro-convulsant mechanisms of HMGB1 and IL-1β

A fast signalling pathway has been characterised showing that IL-1R1 co-localises with the NR2A/B subunit of the NMDA receptor and IL-1 $\beta$  activation of this receptor results in phosphorylation of the NR2B subunit via Src kinases resulting in increased neuronal Calcium (Ca<sup>2+</sup>) influx (Viviani et al., 2003). This fast signalling pathway has more recently also been described for HMGB1 (Maroso et al., 2010). Activation of this pathway in vivo using ceramide to activate Src kinases mimics the IL-1β-mediated exacerbation of kainate-induced seizures, whereas, inhibition of Src kinases has the opposite effect (Balosso et al., 2008). The disulphide inflammatory form of HMGB1, but not its fully-reduced form, acts via TLR4 receptors to exacerbate kainate-induced seizures in a similar manner to that of IL-1β (Balosso et al., 2014). TLR4 receptors colocalise with NR1 and NR2B subunits and disulphide HMGB1 enhances NMDAmediated Ca<sup>2+</sup> influx via neutral sphingomyelinase and Src kinases mediating phosphorylation of NR2B (Balosso et al., 2014). Thus activation of this fast signalling pathway has been proposed as one mechanism for the pro-convulsive effects of both IL-1β and HMGB1. Other potential excitotoxic mechanisms include increasing astrocytic glutamate release, reduction of glutamate uptake and increasing the translocation of AMPA receptors into neuronal membranes (Ye and Sontheimer, 1996, Viviani et al., 2003, Bezzi et al., 2001).

#### **1.7.4 HMGB1 as a pharmacological target**

A number of successful strategies have been shown to inhibit HMGB1 in various experimental pre-clinical models, including polyclonal and monoclonal antibodies (Yang et al., 2004), competitive inhibition with the truncated HMGB1 A-Box (Maroso et al., 2010), recombinant soluble thrombomodulin (Kudo et al., 2013), and selective alpha7-nicotinic acetylcholine receptor agonists (Wang et al., 2004) (figure 1.8). Indeed, nicotine has been shown to inhibit the NF-kB pathway and to suppress HMGB1 release from human macrophages (Wang et al., 2004). Antagonists that neutralize HMGB1 have also demonstrated considerable success in pre-clinical models of various diseases, including severe sepsis, arthritis, colitis, trauma and cancer. For example, administration of anti-HMGB1 antibodies, even where delayed by up to 24 hours following caecal perforation in rodents, rescues the animals from otherwise lethal septicaemia (Qin et al., 2006, Yang et al., 2004). Treatment with HMGB1 antagonists has proven neuroprotective effects in animal models of stroke and head trauma. Following a 2-hour period of middle cerebral artery occlusion (MCAO) in rats, immediate and 6-hour post ischemia delivery of anti-HMGB1 monoclonal antibody (MAb) significantly improved the neurological deficit as defined by rota-rod tests (Liu et al., 2007). Microinjection of short hairpin RNAi for HMGB1 into the rats striatum after MCAO occlusion inhibits neuronal death and the expression of inflammatory mediators including IL-1 $\beta$ , inducible nitric oxide (iNOS), TNF- $\alpha$  and cyclooxygenase-2 (COX-2) 24-hours after occlusion of the MCA (Kim et al., 2006).



Figure 1. 8 High Mobility Group Box-1 (HMGB1) can be targeted to potentially block the pro-inflammatory cytokine cascade. Figure adapted from (Lee et al., 2014) HMGB1: High Mobility Group Box-1; Ab: Antibody; RAGE: Receptor for Glycation End Products; TLR4: Toll-like Receptor 4; TLR2: Toll-like Receptor 2

In the traumatic brain injury (TBI) rat model, inhibition of HMGB1 with ethyl pyruvate significantly improved the degree of resultant cognitive impairment, as determined by the beam walking performance of the rats pre and post injury (Su et al., 2011). A number of successful anti-inflammatory strategies, that directly or indirectly ameliorate HMGB1, have proven anti-seizure effects in preclinical models of seizure and epilepsy (table 1.7)

Taken together, the evidence suggests that HMGB1, in conjunction with other essential inflammatory mediators including IL-1 $\beta$  and the inflammasome complex, is a critical mediator involved in the epileptogenic process that can exacerbate

Model	Anti-	Target	Timing (relative	Outcome	Ref
	inflammatory	10.800	to brain insult)		
	, therapy				
Kainate seizure; rat	IL-1Ra	IL-1R1	Pre- and post-	Reduction in seizure number and time in EEG seizure activity.	(Vezzani et al., 2002)
Electrical self-sustained SE; rat	IL-1Ra	IL-1R1	Pre- and post-	Reduction in seizure behaviour score.	(Vezzani et al., 2002)
Kainate seizure; rat	Pralnacasan	Caspase-1	Pre-	Reduction in seizure-induced IL-1β.	(Ravizza et al.,
				Delay in seizure onset	2006e)
				Reduction in seizure number and time in EEG seizure	
				activity.	
	VX-765	Caspase-1	Pre-	Delay in seizure onset.	
				Reduction in seizure number and time in EEG seizure activity.	
Kainate and bicuculline	Box A	HMGB1	Pre-	Delay in seizure onset.	(Maroso et al.,
seizure models;	LPS-Rs	TLR4		Reduction in seizure number and time in EEG seizure	2010)
mouse	Сур			activity.	
Kainate epilepsy	Box A	HMGB1	Post-	Transient (2 hour) reduction in number and frequency of	(Maroso et al.,
model;	LPS-Rs	TLR4		spontaneous seizures.	2010)
mouse					
Pilocarpine and	IL-1Ra and VX-	IL-1R1 and	Post-	Reduction in IL-1 $\beta$ expression in astrocytes and cell loss in	(Noe et al.,
electrical model of	765	caspase-1		rat forebrain.	2013)
epilepsy; rat				Frequency and duration of spontaneous seizures unaffected.	

 Table 1.7 Anti-inflammatory therapies in experimental seizure models.

Lithium-pilocarpine- induced SE; rat	IL-1Ra and CAY 10404	IL-1R1 and COX-2	Concomitant	Acute delay in seizure onset and decreased neuronal death. Reduction spontaneous seizure frequency, mossy fibre sprouting.	(Kwon et al., 2013)
KA-induced SE; mouse	A438079	P2X7R	Pre- and post-	Reduction in EEG seizure activity and seizure behaviour score.	(Jimenez- Mateos et al., 2012)
KA-induced SE; mouse	A438079	P2X7R	Pre	Reduction in time in EEG seizure activity.	(Engel et al., 2012)
	Brilliant blue G	P2X7R	Pre	Reduction in time in EEG seizure activity. Reduction in SE-induced IL-1β. Reduction in reactive microglia. Reduction in cell death	
	anti P2X7R Ab	P2X7R	Pre	Reduction in time in EEG seizure activity.	-
	A438079	P2X7R	Post- (15 mins)	Reduction in time in EEG seizure activity and total power.	
	A438079 and lorazepam	P2X7R	Post- (1 h)	Reduction in time in EEG seizure activity and total power. No effect on time in seizure activity when administered alone.	
Pilocarpine seizure model; mouse	Carbenoxolone or probenecid	Pannexin1	Pre	Increase in seizure behaviour score and power.	(Kim and Kang, 2011)
	OxATP, A438079 OR A740003	PZX7R	Pre	Increase in seizure behaviour score and power.	
KA-induced SE; mouse	MFQ	Pannexin1	Pre-	Reduction in seizure behaviour score.	(Santiago et al., 2011)

IL-16: Interleukin-16; IL-1Ra: Interleukin-1 receptor antagonist; IL-1R1: Interleukin-1 receptor 1; LPS-Rs: Rhodobacter sphaeroides lipopolysaccharide; Cyp: cyanobacterial lipopolysaccharide; HMGB1: High mobility group Box-1; TLR4: toll-like receptor 4; SE: status epilepticus; P2X7R: P2X7 receptor seizures and be targeted to modulate seizure expression. As such, it is an attractive candidate as a biological marker (biomarker) of the epilepsy disease process.

#### 1.8 Biomarkers

Biomarkers have been defined as "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids" (Hulka, 1990). More recently, a National Institute of Health working group broadened this definition to include "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention" (Biomarkers Definitions Working, 2001).

#### **1.8.1** Potential uses

Biomarkers have myriad potential clinical uses, particularly for drug development. They can be used to measure both normal and pathogenic biological processes, for diagnosis and prognosis, to monitor on and off-target effects of medical interventions and to stratify patient populations into those most likely to respond to treatment. They are relevant to the entire drug development process, from pre-clinical safety indications throughout early drug development trials in small populations to screening of large populations for safety signals post-marketing (figure 1.9.)

#### **1.8.2** Ideal characteristics

The ideal qualities of a biomarker include sensitivity, the proportion of positive responses that a biomarker correctly identifies, and specificity, the proportion of negative responses that a biomarker correctly identifies as negative (Parikh and Ramachandran, 2007). In reality, biomarker development often involves a trade-off between the two. It should ideally be present in an accessible compartment such as blood or urine. It should demonstrate low baseline variability in health with a large dynamic range of quantification such that changes in levels are easily detectable. Ideally, measurable by a high throughput, simple technical analysis that is point-of-care. The analysis should be cost effective (Parikh and Ramachandran, 2007). For drug development especially, translational biomarkers are highly sought after wherein the sequence homology between the pre-clinical species and humans is closely related. The Food and Drug Administration (FDA) issues specific guidance for biomarker

qualification (Food and Drug Administration, 2011) in the form of the biomarker qualification programme. The aim is to ensure that potential biomarkers are fit-forpurpose.



Figure 1.9 Biomarkers are relevant to the whole developmental pipeline for new drug candidates.

#### **1.8.3** Epilepsy biomarkers

Biomarkers in epilepsy would have many potential uses:

- To predict the development of epilepsy following brain insult
- To predict the development of epilepsy following first seizure
- To measure progression
- To predict drug resistance
- To predict susceptibility to adverse reactions to AEDs

Currently, in terms of efficacy, the epilepsy field suffers from a lack of biomarkers able to reliably stratify at or near diagnosis, those with drug resistant epilepsy and those with an epilepsy that will respond to antiepileptic drugs. Biomarkers of early epileptogenesis are also difficult and costly to discover. Even after severe epileptogenic brain insults, only a small proportion of individuals will go on to develop epilepsy, a process which may take more than 10 years to become apparent. As a result, prospective studies in patients with a brain insult have not been undertaken as they are complex to undertake and prohibitively expensive. Nonetheless, biomarkers able to identify a high-risk group for development of epilepsy post-brain insult are particularly sought after. Such a biomarker could enrich trial populations by including only those most likely to develop epilepsy. The ideal situation would be the identification of a panel of biomarkers charting the entire epileptogenic process covering the immediate post-insult epileptogenic period through to preictal/ictal/post-ictal and interictal phases. This will involve a combination of pre-clinical models and human patient studies. The advent of large-scale imaging technologies and clinical neurophysiology, along with genomics, proteomics, and metabolomics means the field of biomarker discovery and validation is likely to change in the near future, from isolated biomarker identification to panels of biomarkers.

#### **1.8.3.1** Neuroimaging biomarkers of inflammation

Activation of astrocytes and microglia in patients with intractable epileptic encephalitis (Kumar et al., 2008; Banati, 2002) and focal cortical dysplasia (Butler et al., 2013) has been successfully shown using positron emission tomography (PET) using 11C-PK11195, a marker of activated microglia. However, this tracer may not prove sensitive enough for the investigation of subtle inflammatory processes, such as that observed in TLE with hippocampal sclerosis (Banati, 2002). The novel radioligand 11C-PBR28, for detection of the inflammatory marker translocator protein (TSPO), demonstrated increased uptake and radioactivity in the seizure focus in patients with temporal lobe epilepsy (Hirvonen et al., 2012). This certainly requires further examination in a larger cohort to correlate PET imaging with the neuropathological findings at epilepsy surgery in order to elucidate the clinical utility of this modality as a biomarker.

To conclude, drug resistance in epilepsy is a significant clinical and societal problem, the causes of which remain poorly understood. There is an unmet clinical need to find new ways to identify early those at greatest risk of developing drug resistance. Inflammation increasingly appears to play a critical role in epileptogenesis. Novel inflammatory biomarkers such as HMGB1, that are intricately involved in the disease process itself, may represent a means to allow recognition of appropriate patient populations who might benefit from anti-inflammatory or immunomodulatory interventions.

#### 1.9 Aims of the thesis

In order to assess whether HMGB1 can be developed as a useful, reliable, mechanistic biomarker for stratification of drug-resistant epilepsy the aims of the thesis were fivefold:

- To determine whether circadian rhythmicity affects expression of peripheral blood HGMB1 in healthy volunteers and to examine whether gender or ethnicity impacts upon peripheral blood HMGB1.
- 2. To determine the concentration of, and relationship between, HMGB1 in paired serum and CSF samples from differing pathologies.
- 3. To examine changes in HMGB1 expression in the mouse brain following KA-SE in a novel, non-surgical multiple-dosing model. To examine the contribution of the acetylation and redox isoforms of HMGB1 to KA-induced epileptogenesis and the time course expression of serum HMGB1 following KA-induced SE by quantification of the different molecular forms.
- 4. To characterize total HMGB1 and both acetyl and redox isoform expression in the rodent brain and serum following both single isolated seizure and spontaneous epileptic seizure.
- 5. To compare baseline blood total HMGB1 between patients with well-controlled (seizure-free) epilepsy and those with drug-resistant epilepsy and recent seizures. To characterize acetyl and redox isoforms of HMGB1 in blood in patients with well-controlled and drug-resistant epilepsy and to characterize changes in serum HMGB1 relative to timing of seizures.

## 2

# Assessment of Circadian Rhythmicity in Peripheral Blood High Mobility Group Box-1: A Healthy Volunteer Biomarker Study
#### 2.1 Introduction

#### **2.1.1** Biomarker characteristics

Biomarkers have been defined as "cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells, or fluids" (Hulka, 1990). The ideal biomarker should display a number of key characteristics. It should be sensitive and specific to the organ or disease state of interest. Ideally it should be present within an accessible tissue (blood, urine) requiring only non-invasive sampling. There should be a large dynamic range allowing detection between states (disease/health, treatment groups) with low baseline variability in a healthy population. Analysis should be cost effective and applicable to any laboratory. In addition, translational biomarkers are able to bridge the preclinical-clinical gap and are particularly sought after for early clinical safety assessment of new therapeutics (table 2.1). Biomarkers also provide a method for homogenous classification of a disease subtype. A biomarker may be described as "mechanistic" if it is in some way implicated in the causal pathway of the disease. It may not be the only determinant but it is strongly associated with the disease process. There are a number of intrinsic characteristics of a biomarker that are essential to its validity. These include knowledge of its background in a general population and an estimation of inter- and intra-individual variability.

A hierarchical distinction exists between a biomarker and a surrogate marker or endpoint. The working definition of a surrogate marker is "a biomarker that is intended to serve as a substitute for a clinically meaningful endpoint and is expected to predict the effect of a therapeutic intervention" (Hunter et al., 2010; Biomarkers Definitions Working, 2001). Not all biomarkers will meet the stringent criteria that are required for surrogate endpoints (Fleming, 2005) including that (a) The surrogate endpoint must be correlated with the true clinical outcome; and (b) the surrogate endpoint must fully capture the net effect of treatment on clinical outcome (Fleming and DeMets, 1996). Biomarkers are particularly sought after in the pharmaceutical industry, the main attraction being the integration of biomarkers into the drug development pipeline with the aim to stop less promising compounds earlier (prior to movement into the costly phase of clinical development, phase III) (Bingham et al., 2006). This would optimize the total cost of drug development. Variability remains a major concern in biomarker utility. Validation is the process of assessing the biomarker and its measurement performance characteristics, specifically whether measurement methods provide accurate and reproducible data (Wagner, 2002). Qualification is the process of linking a biomarker both with a biological process and a clinical end point (Wagner et al., 2007; Wagner, 2002). The FDA issues specific guidance for biomarker qualification (Food and Drug Administration, 2011) in the form of the biomarker qualification programme, which was designed around the Interdisciplinary Pharmacogenomic Review Group, along with expert input from various different FDA centres, including the Centre for Drug Evaluation and Research, The National Centre for Toxicological Research and the Centre for Biologicals Evaluation and Research, among others. The aim is to ensure that potential biomarkers are fit-for-purpose (Lee et al., 2006). The process involves three stages towards acceptance under regulatory guidance: exploratory, probable valid and known valid or "fit-for-purpose" (Goodsaid and Frueh, 2007a; Goodsaid and Frueh, 2007b). Biomarker qualification is considered to be a multi-step process that is continuous and updated as new information becomes available. A number of different strategies have been proposed and are delineated by regulators led by the FDA (Goodsaid et al., 2008).

One important step in establishing the validity of a biomarker test and furthermore, its clinical utility involves assessment of inter-individual variability within a healthy population, including circadian variation.

# 2.1.2 High Mobility Group Box-1 (HMGB1)

A comprehensive description of HMGB1 can be found in chapter 1, section (1.6.3.2). HMGB1 is a 25kda ubiquitous, highly evolutionarily conserved protein with distinct intra and extra-cellular functions. In all cells, HMGB1 continuously shuttles between the nucleus and cytoplasm. Within the nucleus, HMGB1 bends DNA and regulates transcription (Avoli et al., 1996). Released passively from necrotic cells, HMGB1 can also be actively secreted upon inflammatory activation of the cell. In the extracellular milieu, dependent upon specific post-translational modifications, it can function as a chemoattractant (Schiraldi et al., 2012) or pro-inflammatory cytokine (Leite et al., 1990).

#### Table 2.1 Ideal performance characteristics of a biomarker

Characteristics of an ideal biomarker
Safe to measure
Easy to measure
Low cost to measure
Cost of follow-up test is relatively low
Consistent across genders and ethnic groups
Displays low baseline variability in health
Displays large dynamic range of quantification
Translational between preclinical species and humans

Table expanded from (Parikh and Ramachandran, 2007)

In humans, several disease states have been associated with high levels of peripheral blood HMGB1 including sepsis (Loscher, 2002; Sunden-Cullberg et al., 2005), disseminated intravascular coagulation (Coulter et al., 2002), acute coronary syndromes (Richter and Loscher, 2002; Roder et al., 1994; Sofroniew, 2009) and various cancers (Ledur et al., 1995; Blackburn et al., 1991; Guglielmo-Viret et al., 2007; Swanson et al., 1999). These studies suggest that HMGB1 has potential as a sensitive and specific biomarker for stratification of a sub-population of patients.

# 2.1.3 HMGB1 in healthy individuals

At present, the reference values for HMGB1 in health are not known. The International Federation of Clinical Chemistry (IFCC) recommends the mean value should be obtained from a minimum of 120 healthy subjects (Solberg, 1987). The largest study of HMGB1 levels in a healthy Japanese population over 40 years of age (n=626) provides a mean value of 1.65 ± 0.04ng/ml (Fukami et al., 2009). Variation in baseline HMGB1 may have important implications for the timing of blood sampling when considering HMGB1 as a predictive biomarker. At present, patterns of circadian variation in HMGB1 levels have not been established.

# 2.1.4 Circadian rhythmicity

Circadian rhythms are endogenous physiological cycles with a recurring periodicity of approximately 24 hours. They are generated by the suprachiasmatic nucleus which is

located in the anterior hypothalamus and is known as the 'biological pacemaker' (Golombek and Rosenstein, 2010). Circadian rhythms affect the expression of approximately 2 to 10% of genes in mammals (Storch et al., 2002). Blood-borne biomarkers are subject to variability in both sample processing and natural biological processes. In addition, circadian rhythmicity and diurnal variation is an important source of systematic variability, being consistent in nature as opposed to the random fluctuations around the mean inherent to steady-state periods.

#### 2.1.4.1 Circadian rhythmicity of HMGB1

The circadian clock in eukaryotic cells is regulated by several proteins which form a feedback loop. These 'clock-control' genes vary depending upon tissue type. The target proteins are activated by the core-clock promoters by binding to specific DNA sites in their regulatory regions (Gekakis et al., 1998). Binding sites for non-histone proteins, including the HMG-family, have been found to be overrepresented in promoters of clock-controlled genes (Gekakis et al., 1998).

In plants, expression of the HMGB1 gene is regulated by both an endogenous circadian mechanism and the light/dark cycle (Zheng et al., 1993). In rat photoreceptor nuclei, HMGB1 exhibits diurnal and circadian-clock dependent changes, with peak levels observed during the light phase. This protein fluctuation is sustained in complete darkness (Hoppe et al., 2007).

#### 2.1.4.2 Circadian rhythmicity of HMGB1 in epilepsy

It has been demonstrated in studies of human focal epilepsy that endogenous circadian rhythms may cause a day/night pattern in seizures in temporal lobe epilepsy (Pavlova et al., 2004; Quigg et al., 1998). In a study of 90 seizures (41 occurring in temporal lobe epilepsy and 49 in extra-temporal lobe epilepsy) 50% of seizures occurring in the TLE group occurred between the hours of 15:00 and 19:00 (17% would be expected by chance, p<0.006) (Pavlova et al., 2004).

#### 2.1.5 Analytical validity in biomarker measurement

Another important step in determining accuracy in biomarker trials concerns the use of accurate analytical methods. Accurate quantification of a particular biomarker in any biofluid requires the use of a method with appropriate analytical validity. Specifically, the accuracy, reproducibility and reliability of the method needs to be adequately established. To develop and validate a biomarker test, several critical issues must be met. The current gold standard for measurement of total HMGB1 is the commercially available ELISA from Shino-Test Corp., Sagamihara, Japan. The manufacturer's assessment of precision can be found in table 2.2 (full data sheet appendix).

HMGB1 ELISA	
Dynamic range	2.5 - 80 ng/mL
Sensitivity	1 ng/mL
Specificity	Cross-reaction with HMGB2 is < 2%
Reproducibility	Intra-assay coefficient of variation is < 10% (n = 8) Inter-assay coefficient of variation is < 10% (n = 8)
Recovery	80 - 120%
HMGB1: High mobility group box-1; ELISA: I	Enzyme-linked immunosorbent assay

2.1.1

# 2.1.6 Rationale

In the presence of circadian variability, adjustments may need to be made in the interpretation of biomarker results and standardization of sampling time may be necessary. Particularly for prognostic clinical biomarkers, where patient sampling may be undertaken at any time during the day or night, it is essential that the presence of circadian rhythmicity be confirmed or excluded.

The aims of this study were:

- 1. To determine whether circadian rhythmicity affects expression of peripheral blood HGMB1 in healthy volunteers.
- To examine whether gender or ethnicity impacts upon peripheral blood HMGB1.

# 2.2 Methods

Research participants (1-4) were recruited at ICON developmental solutions Manchester, United Kingdom. ICON is a contract research organization specializing in early phase clinical trials. The site suspended all studies in June 2013, following which study recruitment then relocated to ICON developmental solutions, San Antonio, Texas, United States of America (USA) in October 2013.

# 2.2.1 Study population – Recruitment Process

Subjects consisting of healthy male and female volunteers aged 18-45 years were recruited from the ICON Developmental solutions volunteer panel.

The inclusion criteria were:

- Subject was willing and able to give written informed consent
- Healthy male or female subjects between 18 and 45 years of age inclusive
- Body weight between 50 and 100 kg
- Body mass index between 18 and 32 kg/m2

The exclusion criteria were:

- Subject had a clinically significant abnormal medical history or physical examination.
- Subject had a history of febrile illness within 4 weeks prior to admission.
- Subject had a clinically significant abnormal laboratory test at screening.
- Subject had admission vital signs outside normal limits (defined by blood pressure >140 systolic/90 diastolic, resting pulse >100 or <50 beats/minute, resting respiratory rate > 20 breaths/minute, oral temperature >37.3 °c)
- Current smoker or history of smoking within the 6 months prior to the screening visit.
- Positive test for hepatitis B, hepatitis C or human immunodeficiency virus
- Donated or lost more than 100 millilitres of blood in the 4 weeks prior to screening or donated or lost 500 millilitres of blood in the 3 months prior to

screening. Subjects were also willing not to donate blood for 3 months after the study.

 Consumption of any prescription drug, non-prescription drugs, vitamin or dietary supplements within 2 weeks (whichever is longer) prior to study admission. Herbal supplements were discontinued at least 4 weeks prior to admission to clinical unit.

# 2.2.2 Sample size calculation:

To date, no intensive sampling circadian biomarker studies have been published in man. Most circadian studies undertake bi-daily sampling (morning and evening). This study was the first of its kind to examine 3-hourly samples across a complete 24-hour period. As such, a sample size calculation could not be performed based on published studies. An estimation of 24 individuals for an initial pilot study was undertaken with a view to increasing recruitment should it be felt necessary. Post-study analysis of the samples confirmed that 24 individuals were sufficient and no further patients were recruited.

# 2.2.3 Ethical Approval:

Ethical approval for this study was initially granted by the North West Research Ethics Committee Manchester Central (12/NW/0374). Approval covered collection of both blood and urine from the patients up to a maximum of 400mls/blood over a 24 hour sampling period. Approval covered analysis of blood for any potentially novel biomarker and/or inflammatory marker. Following relocation of the study to the San Antonio site in the USA, approval was granted by the institutional review board (LLC 0327/014).

# 2.2.4 Study Schedule

# 2.2.4.1 Pre-study screening visit

Healthy subjects (n=24) identified from the register were invited to attend a pre-study screening visit within three weeks of potentially starting the study. A trials physician at ICON explained the study and obtained written consent. Subsequently, eligibility screening was undertaken according to the protocol and screening bloods to confirm HIV and Hepatitis B and C serology were negative.

# 2.2.4.2 Study restrictions

The restrictions for the volunteers enrolled onto the study were as follows:

- Diet: All subjects were provided with standardized meals and snacks at set times during the inpatient admission.
- Concurrent Medication: Any prescription/non-prescription drug, vitamin or dietary supplement was forbidden within 2 weeks of admission to the clinical unit. Herbal supplements were discontinued at least 4 weeks prior to admission.
- Alcohol and caffeine: Subjects were required to refrain from alcohol and caffeine consumption from 48 hours prior to the screening visit and admission to the clinical unit.
- Smoking: A minimum of 6 months non-smoking prior to the screening visit was required.

# 2.2.5 Main Study Period

The main study period was undertaken at the ICON Development Solutions Clinical Unit located on the Manchester Royal Infirmary hospital site (subjects 1-4) or at the Phase 1 unit, San Antonio Texas USA.

On the day before sampling, subjects were admitted to the unit at 13.00 where suitability and consent were confirmed. Subjects were provided with a standardized meal at 18.00 followed by a standardized snack at 21.00 following which the subjects underwent a 10-hour overnight fast. On the day of the study a cannula was inserted at 07:00 hours from which blood samples were drawn every 3 hours over a 24 hour period between 07:00 and 22:00 and then again at 00:00 on day 1 and 04:00 on day 2. Subjects were discharged at 08.00 on day 2.

# 2.2.6 Sample Processing

The cannula was flushed with 2 millilitres of 0.9% saline prior to each sample being taken and 0.5mls of blood was withdrawn and discarded. Samples for serum were collected in 9ml plain tubes, which were centrifuged within 15 minutes of collection or stored upright overnight at 4°C and centrifuged the following morning. Serum was

transferred in dry ice with temperature monitoring to the Wolfson centre laboratories, University of Liverpool for analysis.

#### 2.2.7 Serum HMGB1 quantification by ELISA

Preparation of standards solution, detection antibody solution and read buffer was performed in accordance with the manufacturer's instructions (Shino-Test Corp., Sagamihara, Japan). All reagents from the kit were allowed to warm at room temperature prior to preparation. The standard stock (320ng/ml) was reconstituted with sample diluent. A series of standards were prepared by serial dilution of the calibrator by adding 100µl of the calibrator to 300µl of diluent, vortex-mixing, and repeating the process by adding 100µl of standard to 100µl of diluent and repeating 5 times to generate a total of 7 standards. The standards were left on ice for 10 minutes. Following thawing on ice, samples were centrifuged at 2000xg for 1 minute. The 96-well plate was coated with sample diluent to which 10µl of serum sample was added in duplicate. The plate was sealed and samples were incubated overnight at 37°C for 20 hours. Plates were washed 5 times in wash buffer (400 $\mu$ l/well) and air dried. Detection antibody solution (100µl/well) was added for 2 hours at room temperature. Following the subsequent washing step, substrate solutions were added in equal parts (100µl/well) and incubated at room temperature protected from light with foil seal for 30 minutes and the plate read at 450nm on Beckman Coulter version (DTX 880) Multimode Detector using multimode detection software. Results were fitted to the standard curve.

The methodological characteristics of the ELISA have been assessed both by the manufacturers and external researchers previously (appendix, (Antoine et al., 2009)). Intra and inter-day imprecision and dilutional linearity were within an acceptable range. According to the manufacturer, the limits of intra and inter-assay precision range from 0.2-80ng/ml with CV <15%.

#### 2.2.8 Statistical Analysis

Statistical computations were performed using GraphPad Prism (GraphPad Software, San Diego, CA) and SPSS software. The mean (± standard error of the mean) concentrations for each time point were calculated. To test for variability in levels both

between individuals and between time-points, data were analysed using one-way repeated measures ANOVA. Mauchly's test of sphericity was undertaken to check that the assumption of sphericity was not violated. The analysis was undertaken both with and without including covariates to adjust for gender and race.

# 2.3 Results

# 2.3.1 Subject Characteristics

A total of 24 healthy non-smoking subjects (table 2.3) underwent serial sampling over a 24-hour period. Subjects ranged in age from 19 to 46 years (mean 34 years). The individual characteristics of the subjects are outlined in table 2.4.

#### Table 2.3 Demographics of the study population.

Characteristics	Subjects (n=24)
Male/Female	14/10
Caucasian/African American	16/8
Age, years	34 (± 1.9), range 19-46
Systolic BP mmHg	115 ± 2.2
Diastolic BP mmHg	74.4 ± 1.8
Heart rate, beats/minute	71 ± 2.6
HMGB1 ng/ml	1.81 ± 0.05

The data are shown as the mean values ± standard error of the mean. BP: Blood pressure.

# 2.3.2 Circadian Variability

The overall mean HMGB1 concentration was  $1.81 \pm 0.05$  ng/ml (table 1-3). The median values and interquartile ranges plus minimum and maximum values for each subject are expressed as box plots (figure 2.1.A.) Individual profile plots are shown in figure 2.1.B. 5/24 subjects had a single time point excluded due to sample haemolysis.

There was no significant within-subject variability in HMGB1 concentration demonstrated across the sampling time frame from 07:00 on day 1 until the last sample at 04:00 on day 2 (Table 2.5), either when adjustments were made for race

(Caucasian: African-American) and gender (male: female) (p-value: 0.72) or when no adjustments were made (p-value: 0.55). In both analyses, Mauchly's test indicated that the assumption of sphericity had not been violated.

# 2.3.3 Relationship between baseline HMGB1 and other clinical variables of health.

A number of baseline safety blood tests were taken alongside the early morning HMGB1 sample together with measurement of blood pressure. Linear regression analyses comparing these variables are shown in figure 2.2. No significant association between HMGB1 and any of the clinical variables was identified.



Figure 2.1 A. Box and whisker plots depicting high mobility group box-1 (HMGB1) concentrations over time. Each box represents the 25th and 75th percentiles. Lines outside the boxes represent the minimum and maximum limits. Lines inside the box represent the median with the dot representing the mean. Dotted line represents overall mean. B. Individual profile plots depicting baseline variability in HMGB1 concentrations across a 24 hour sampling period.

Sex	Age	Race	Medical History	Alcohol (units/ week)
F	43	Caucasian	Caesarean-section childbirth 1995, 1997 with tubal ligation 2002	0
F	43	Caucasian	None	0
Μ	45	African American	Appendicitis and appendicectomy 1975, left inguinal hernia repair 2007	0
F	24	African American	Menstrual cramps 2009, vaginal childbirth 2007, 2008, 2009,2012	0
Μ	25	African American	None	0
Μ	46	Caucasian	None	3
Μ	28	Caucasian	None	3
F	41	Caucasian	None	0
Μ	41	Caucasian	Crushed left zygomatic arch 1991 - surgical repair 1991, fractured left wrist 1985	6
Μ	28	African American	Right elbow laceration 2001 with skin graft	6
Μ	43	Caucasian	Bowel perforation due to gunshot wound to abdomen 1989	0
Μ	27	Caucasian	Headaches 30.08.2013	0
Μ	39	Caucasian	None	0.5
Μ	26	Caucasian	None	0
F	28	Caucasian	None	0.5
F	19	African American	None	0
Μ	45	Caucasian	None	0
F	21	African American	None	0
Μ	21	Caucasian	Skin abrasions 2013	1
Μ	44	Caucasian	None	4
Μ	28	African American	Adenoiditis 1998, tonsillitis 1998, tonsillectomy 1998	1
F	39	Caucasian	Ventricular septal defect 1979, open heart surgery 1979	4
F	38	Caucasian	Acne face 1990 - 2000, caesarean-section child birth 2011 and 2013	0
F	38	African American	None	1

 Table 2.4 Individual patient characteristics and background medical history

	Mean [HMGB1]			
Time point	ng/ml	S.E.M	n	
Day 1 07:00	1.868	0.14	24	
10:00	1.610	0.14	24	
13:00	1.797	0.15	24	
16:00	1.788	0.15	24	
19:00	1.645	0.12	24	
22:00	1.735	0.14	24	
00:00	1.771	0.14	24	
Day 2 04:00	1.773	0.16	24	

Table 2.5 Table depicting the mean HMGB1 concentration per time point.

*The data are shown as the mean values* ± *standard error of the mean.* 

#### 2.4 Discussion

The intensive sampling data from this study shows that HMGB1 concentrations in healthy subjects are not subject to any significant circadian variability. Furthermore, there is no significant degree of inter or intra-individual variability in the biomarker. It should therefore be acceptable to obtain HMGB1 biomarker samples at any time of day without the need to adjust for time, gender or race (applicable to Caucasian and African Americans). The mean value for HMGB1 in health in this cohort is in keeping with the largest analysis of serum HMGB1 in health in 626 Japanese subjects where the mean value was defined as  $1.65 \pm 0.04$  ng/ml. The ongoing BIOPAR study (Antoine et al, unpublished data) examining HMGB1 in 200 healthy Caucasian subjects had a mean level of  $1.18 \pm 0.61$  ng/ml (60% female, mean age 35 years), in keeping with the findings of the present study. When inter-individual variation in a biomarker is large in comparison to intra-individual variation, analysis of paired samples (before, during and after the event/exposure) is required to greatly enhance the power of the biomarker to detect exposure. However, this is not necessary for HMGB1 due to its low variability.



Figure 2.2 No significant association between clinical variables and baseline HMGB1 (early morning samples.) Kendall's Tau correlation is illustrated by the solid line, with the corresponding correlation co-efficient reported.

Variability, both biological and analytical, limits the clinical utility of candidate biomarkers. Biological variation, the random fluctuation of a constituent of the human body around the homeostatic setting point, has two components: within-subject (intra-individual) and between-subject (inter-individual) variation. Sources of intraindividual variation include ageing, gender, body weight, diet, exercise, circadian rhythms, sleep cycles and pathological processes (Ricos et al., 2004). In order to assess the degree of intra and inter-individual variability in peripheral blood HMGB1, a prospective cohort of 24 healthy volunteers was undertaken across a 24 hour period (Chapter 2.) It was possible to conclude that the variation, both within and between subjects, was minimal and did not meet statistical significance at any time point, regardless of adjustments made for gender and ethnicity. The study was limited by small sample size (n=24) which had to be estimated as there were no similar studies available in the literature that utilise an intensive sampling time frame (3 hourly). Acknowledging the small sample size, a post-hoc power calculation was undertaken to illustrate the available power, using g\*power sample size software (Faul et al., 2007). For the power calculations, variability between time-points was assumed to be approximately 0.005 (estimated from the data) and variability within time-points to be approximately 0.48 (again estimated from the data). It was also necessary to estimate correlation between pairs of observations from each subject, which is challenging: this was estimated, based on the data, to be in the range of 0-0.6. On this basis, power was estimated to be 13% if correlation was assumed to be low (=0) and 32% if correlation was assumed to be high (=0.6). Therefore, to achieve 80% power, a sample size of 162 subjects would be required, assuming low correlation, and a sample of size of 66 subjects assuming high correlation. Therefore, the sample size of 24 may be insufficient to categorically exclude the impact of circadian variability. Nevertheless, the sample size calculation is an estimate, the intensive sampling undertaken in this study is novel, and to undertake a study with this many patients in a controlled setting such as a clinical research facility would be prohibitively expensive.

Within-subject variation is known to be much smaller than between-subject variation for the majority of substances assayed in laboratory medicine (Fraser, 2004). The reference change value (RCV) is a method for expressing the difference in a result from a single individual with respect to his/her previous results, instead of on populationbased values and can be obtained in serial sampling studies (Harris, 1976). It accounts for both analytical and biological variation. The RCV tells the clinician whether the test result has changed in an important way, beyond the difference expected from the inherent sources of variation (Fraser, 2004). These sources include pre-analytical variation, analytical imprecision (CVA), and within-individual biological imprecision (CVI). The RCV can be calculated as:  $RCV = 21/2 \times Z \times (CVA2 + CVI2)1/2$ , where Z is the number of SDs appropriate to the probability. Unfortunately the RCV for the current study could not be calculated as it requires imputation of the analytical coefficient of variation, as determined by a standardised analysis method and requires a sophisticated laboratory information system (Cooper et al., 2011). It would require experimentation under controlled conditions on serum samples obtained from healthy subjects in order to determine the accurate within-plate and between-plate variability (CV), which was beyond the scope of this thesis. Despite some drawbacks, the RCV is increasingly felt to be a clinically relevant tool to determine variability in daily practice, beyond the conventional diagnostic sensitivity, specificity, and predictive values of positive and the negative tests (Henderson, 1993; Zweig and Campbell, 1993).

Circadian variability in CNS biomarkers is not unheard of. Indeed, significant hour-tohour variability has been demonstrated in Alzheimer's disease (AD) CSF biomarkers, amyloid  $\beta$  protein (A $\beta$ ) A $\beta$ 40 and A $\beta$ 42, in young non-demented participants (Bateman et al., 2007). This variability diminished in healthy older controls and patients with Alzheimer's disease (Slats et al., 2012), demonstrating that consideration of time and subject-specific characteristics are important when developing biomarkers for clinical use. Between-subject and between-laboratory variability hinders the definition of clear-cut-off values for clinical practice and indeed, the variability in CSF biomarkers for AD is thought to be too high to allow determination of a universal cut-off value (Mattsson et al., 2013). Less is known about the variability in CSF biomarkers for Parkinson's disease (PD) including DJ-1,  $\alpha$ -synuclein, tau and  $\beta$ -amyloid peptides. Daily sampling across a 26-hour period in both healthy volunteers and patients with PD identified consistent levels across the patients but that Alpha-synuclein, Abeta 1-42, and Abeta 1-40, but not DJ-1, increased significantly over time (Frasier, 2013). Different pre-analytical procedures between laboratories is reported to be, at least in part, responsible for the high variability found between study centres in the concentrations of both AD CSF biomarkers (Aβ42, total tau and phosphorylated tau) and the PD CSF biomarker (a-synuclein) (Mattsson et al., 2011; Mollenhauer et al., 2010; Sunderland et al., 2003). It is therefore important to consider a standardized protocol for analysis when designing future HMGB1 biomarker trials.

Few studies have examined variability in biomarker expression in peripheral blood during a designated, standardized inpatient stay wherein exercise can be limited (Kong et al., 2006; Hetland et al., 2008). Arguably, inpatient studies examine biomarker levels in a vacuum, with results that may not be generalizable to a population in whom exercise, stress levels and sleep patterns, among other factors, vary. However, initial studies where the objective is to identify within-subject factors are best undertaken under highly regulated conditions, such as the study undertaken here, with follow-up larger scale studies to identify whether such factors are clinically relevant undertaken in outpatient settings.

Age is an important factor to consider, as reports have demonstrated that chronic, low-grade inflammation is linked with the aging process (Bruunsgaard and Pedersen, 2003; Bruunsgaard et al., 2001; Forsey et al., 2003). However, the current study examined a narrow age range (19 to 46 years), a limitation of the majority of studies performed in clinical research units utilising trials registers. Thus, in the future, examination of HMGB1 at extremes of age is ideally required in order to account for this effect.

Albeit a small sample size, the results of this study show that it should be acceptable to obtain serum HMGB1 biomarker samples at any time of day without the need to adjust for time, gender or ethnicity. These findings are particularly relevant to studies of seizure and epilepsy, whereby samples may be obtained at any time, ranging from an early morning epilepsy clinic to a middle-of-the-night emergency department.

Development of minimally invasive biomarker assays for early detection and effective clinical management of disease are highly sought after. For epilepsy in particular, biomarkers that predict onset and progression of the disorder and its response to treatment are currently lacking. The assessment of clinical validity of a biomarker is central to its development. Clinical validity is a measure of the markers diagnostic accuracy, that is, the degree to which it can be used to identify diseased patients from those that are free of disease. This in turn relies on comprehensive and accurate attainment of biomarker values in the healthy state. The data from the present study provide an accurate measure of health for comparison to any disease state.

# 3

# A Comparison of High-Mobility Group Box 1 between Cerebrospinal Fluid and Peripheral Blood

# 3.1 Introduction

Very limited data are available to assess the relationship between peripheral blood and centrally derived CSF levels of HMGB1, particularly in conditions in which there is evidence that the barrier isolating brain from blood, the BBB, is disrupted and therefore a conduit for transfer of the biomarker exists.

# 3.1.1 Cerebrospinal fluid

CSF is a low protein, clear colourless fluid that surrounds the brain and spinal cord. A third is is produced by the epithelial cells of the choroid plexus, the remaining twothirds is bulk flow of extracellular fluid and therefore bathes the brain. Fluid turnover in the brain is slow, 3-4 times per day (Sakka et al., 2011). CSF circulates slowly around the CNS in a pulsatile manner, produced at a rate of around 500-600mls per day and is constantly reabsorbed into the venous circulation across the arachnoid villi (Sakka et al., 2011). CSF cushions the brain and maintains electrochemical homeostasis. The concentration of albumin in the CSF ranges between 7.8-40mg/dL, corresponding to between 0-0.7% of that in peripheral blood (Fischbach, 2003).

# 3.1.2 Blood-brain barrier

The highly controlled and stable microenvironment of the central nervous system is maintained by an anatomical BBB within brain capillaries. The BBB shows much lower passive permeability than for other vascular compartments. Tight endothelial junctions, unfenestrated capillaries and paucity of pinocytic vesicles all contribute to reduced permeability (Redzic, 2011). The highly changeable composition of peripheral blood provides a constant stream of essential molecules and removes metabolic waste from the brain interstitial fluid. Exchange of molecules occurs across the BBB via various influx and efflux transporters (Pardridge, 1983). Ideal qualities for CNS penetration via the CSF include lipid solubility, low ionization or hydrogen bonding and low molecular weight (Pajouhesh and Lenz, 2005).

# 3.1.3 Blood-brain barrier integrity

The degree of permeability of the BBB can be estimated from the CSF/serum quotient for albumin (QALB). The albumin quotient is determined by CSF albumin/serum albumin x 100. If the blood-CSF barriers are intact, the albumin quotient is typically <7x

10-3 (Marshall, 2008). The passage of serum-derived albumin into the CSF across a disrupted BBB causes the quotient to rise. The degree of disruption may be quantified as <0.7%, normal; <2.0%, mild; <5.0% moderate; >5% severe (Janigro, 1999). Inaccuracies can arise when serum and CSF samples are analysed on separate analytical runs as a result of inter-assay variability and hence caution must be exercised that paired samples are analysed within the same run.

#### 3.1.4 CNS Immunity

The CNS is considered an immunologically privileged site owing to the mechanical barrier provided by the CNS and the immunoregulatory characteristics of the resident microglial cells and astrocytes (Galea et al., 2007). Separation of CNS immunity from perturbations in the peripheral immune system serves to protect the limited regenerative capacity of the sensitive brain. CNS immune privilege is not wholly absolute and is limited at extremes of age and during CNS inflammation (Perry et al., 1993; Lawson and Perry, 1995; Anthony et al., 1997). Pro-inflammatory cytokines have been identified in the CSF of multiple neurological conditions in humans including spinal cord injury (Kwon et al., 2010a), multiple sclerosis (reviewed in (Mazzi, 2015), meningitis (Ichiyama et al., 1997; Mustafa et al., 1989; Ohga et al., 1994) and autism spectrum disorders (reviewed in (Xu et al., 2015).

#### 3.1.5 Neurological effects of High Mobility Group Box-1

High mobility group box-1 (HMGB1, described in detail in 1.6.3.2) is a chromatin protein whose varied functions are dictated by post-translational modifications. Critical to the biological activity of the three potential redox isoforms are the highly conserved cysteine residues found at positions 23, 45, and 106. Fully-reduced HMGB1, complexed to CXCL12 and bound to CXCR4, recruits leucocytes and inflammatory mediators to sites of necrosis (Venereau et al., 2012). The main isoform that accumulates in the extracellular space and serum compartment during acute and chronic inflammation is disulphide-HMGB1, wherein a disulphide bond forms connecting Cysteine23 and Cysteine45. The resultant isoform binds TLR4 and activates macrophages/monocytes and other cells to produce cytokines and additional inflammatory mediators (Andersson et al., 2014; Antoine et al., 2014). Finally, in the fully oxidized state, HMGB1 is considered to be non-inflammatory. In the brain, exogenous application of HMGB1 acts as a mediator of fever, anorexia and memory disturbance (Agnello et al., 2002; Mazarati et al., 2011). In addition it has been shown to increase both TNF and IL-1 $\beta$  expression (Agnello et al., 2002).

#### 3.1.6 The relationship between central and peripheral immune

#### compartments.

Strong evidence exists that the BBB is disrupted in both neuroinfection (Quagliarello et al., 1991; Paul et al., 1998; Nizet et al., 1997; Doran et al., 2005; Das et al., 2001) and seizure disorders (David et al., 2009; Seiffert et al., 2004; Tomkins et al., 2007; Tomkins et al., 2008; Pavlovsky et al., 2005) and therefore a conduit for transfer of biomarker exists. HMGB1 has been suggested to be involved in the aetiopathology of the several neurological conditions discussed in detail below. However, whether the source of HMGB1 is the peripheral or central immune system remains to be established.

# 3.1.7 Neuroinfection

#### 3.1.7.1 The immune response to neuroinfection

The innate immune response to neuroinfection is triggered by recognition of highly conserved PAMPs expressed by invading pathogens, most commonly Streptocococcus pneumoniae (Wetherington et al., 2008). TLR activation leads to assembly of multimolecular complexes called inflammasomes. Inflammasomes are defined by expression of specific pathogen recognition receptors, for instance NLRP3 (NLR family, pyrin domain-containing 3) (Strowig et al., 2012). Inflammasomes control the production and release of pro-inflammatory cytokines including activated IL-1 $\beta$  and IL-18 (Davis et al., 2011; Schroder and Tschopp, 2010). Consequently, this leads to the recruitment of blood-borne leucocytes into the leptomeninges via up-regulated expression of multiple leukocyte adhesion molecules (Wetherington et al., 2008). BBB disruption is a pathological feature of bacterial meningitis. The host response to bacterial infection continues even after antibiotics kill the bacteria and is a significant contributor to CNS injury.

#### 3.1.7.2 Bacterial meningitis

In children with bacterial meningitis, significantly higher levels of CSF HMGB1 have been reported compared to aseptic (viral and culture/white cell count negative) forms of the disease (mean difference 36.18, p<0.01) (Tang et al., 2008). In children with suspected meningitis, CSF HMGB1 was significantly higher in bacterial and aseptic cases when compared with febrile seizure and no CNS infection (bacterial 48.9 +/- 63.5 ng/ml vs. 0.16 +/- 0.45 ng/ml, p<0.01; aseptic 4.85+/- 4.59 ng/ml vs. 0.16 +/- 0.45 ng/ml, p<0.05) (Asano et al., 2011). In both studies, HMGB1 correlated with CSF white cell count. Determination of peripheral blood HMGB1 was not undertaken in either study. In 4 adults with pneumococcal meningitis, western blot quantification of CSF HMGB1 revealed large concentrations (4>ug/ml) were present (Hohne et al., 2013). In peripheral blood, a larger study of suspected CNS infection in Malawian children revealed that HIV+ children with serious bacterial infections (SBI) had significantly higher serum HMGB1 than controls (SBI+, n=145 7.6 ng/ml vs. SBI- n=45 6.0 ng/ml, p=0.05) (Carrol et al., 2009).

#### 3.1.7.3 Viral central nervous system infection

In 20 patients with pandemic H1N1 influenza-virus associated encephalopathy, serum HMGB1 was significantly elevated in those with poor outcome compared to those without neurological sequelae (median 17.4ng/ml vs. 6.8ng/ml, p<0.05) and 17 control afebrile children with neurological disorders (5.5ng/ml, p<0.001). In all subjects, the CSF HMGB1 level was <1.0ng/ml (Momonaka et al., 2013). HMGB1 in children with acute non-infectious encephalopathy has not been shown to be elevated (n=8, mean 0.23 +/- 0.44 ng/ml).

#### 3.1.8 CNS immunity and non-infectious pathology

#### 3.1.8.1 Subarachnoid haemorrhage

Subarachnoid haemorrhage (SAH), resulting from bleeding into the subarachnoid space (between the pia mater and arachnoid mater of the brain), usually occurs as a result of a ruptured intracerebral aneurysm. Inflammatory cytokines including interleukin-6 (IL-6), IL-18 and TNF- $\alpha$  have all been shown to be elevated in CSF following SAH (Janigro et al., 1999; Mathiesen et al., 1993; Osuka et al., 1998; Marchi et al., 2007; Nakahara et al., 2009; Zhu et al., 2012). A parallel rise in serum IL-6 was not seen in 12 patients with paired sampling after SAH (Mathiesen et al., 1993), suggesting the source of the cytokine production to be central in this condition. CSF

samples were obtained from the CSF drainage tubes at the site of postaneurysm clips in 39 patients with SAH.

In addition, HMGB1 has also been shown to be significantly elevated following SAH (mean 25.2ng/ml day 3, 22.9ng/ml day 7, 10ng/ml day 14) compared to neurologically normal control subjects in whom CSF HMGB1 was undetectable (Nakahara et al., 2009). However, this study involved patients undergoing craniotomy with drainage tube insertion, which may influence the degree of inflammatory reaction. A series of 303 consecutive patients with SAH revealed that plasma HMGB1 level on admission was a significant predictor of poor functional outcome and mortality after 1 year, inhospital mortality and cerebrovasospasm (Zhu et al., 2012).

#### 3.1.9 CNS immunity and seizure disorders

BBB disruption permits interaction between the peripheral and central immune system. Increasingly, evidence from both experimental models of seizure (Seiffert et al., 2004; Pavlovsky et al., 2005; David et al., 2009; Fabene et al., 2008) and human epilepsy (Marchi et al., 2007) support the notion that disruption of the blood brain barrier (BBB) sets in motion a cascade of downstream events leading to the development of an epileptic focus. Immune cells, including TGF- $\beta$  and various leukocyte adhesion molecules, appear to contribute to the pathophysiology of seizure disorders by mediating BBB breakdown (reviewed in detail in chapter 1.5.1). What is not yet accurately known is whether the disruption occurs before (a prerequisite), during (pathophysiological) or after (a consequence) seizure. Indeed, ultrastructural animal studies and human epileptic tissue reveal that focal epilepsy often develops following localised breakdown of the BBB as a result of traumatic, ischaemic or infectious brain injury (van Vliet et al., 2007a; Oby and Janigro, 2006; Abbott et al., 2006). The contribution of HMGB1 to BBB disruption has not been studied to date.

#### 3.1.10 Rasmussen's encephalitis

Rasmussen's encephalitis (RE) is a rare, CNS inflammatory disorder characterized by intractable seizures, unilateral hemispheric atrophy and progressive neurological deterioration (Rasmussen et al., 1958). The aetiology remains undetermined, although lymphocyte infiltration and microglial nodules are characteristic (Bien et al., 2005),

suggesting a viral component may be responsible; however no pathological agent has ever been confirmed. The majority of seizures are focal motor seizures. Epilepsia partialis continua (EPC), consisting of continuous focal seizures or repetitive focal motor seizures, usually affecting the hands or face, is a common feature and can last for days (Thomas et al., 1977). This eventually leads to a hemiparesis, and it has been suggested that recurrent seizure activity may be the causative factor in the neurological decline (Bien et al., 2002; Bien et al., 2005). The current understanding of RE is that of an immunologically-mediated disease, however the relative contributions of T-cells, B-cells and autoantibodies remains to be determined. The severity of the epilepsy and the degree of deficit may be reduced by the use of corticosteroids and other immunotherapies (Hart et al., 1994). Tacrolimus is an agent directed at T-cells which was tested in an open study of patients with RE and demonstrated that both hemispheric atrophy and the progression of hemiparesis was slower in the treated group compared to untreated controls (Bien et al., 2004). Rituximab is an anti-CD20 antibody that destroys B-cells. A recent case report details a young woman with intractable seizures and RE was rendered seizure free for several months following intravenous rituximab therapy (Thilo et al., 2009). To date, expression of HMGB1 in CSF or peripheral blood has not been examined in this condition.

#### 3.1.11 Idiopathic Intracranial Hypertension

Idiopathic intracranial hypertension (IIH) was described at the end of the 18th century. It is characterised by headaches and nausea resulting from raised intracranial pressure in the absence of an obvious pathological brain condition. Normal adult intracranial pressure is defined as 7.5-20cm H20 (Czosnyka and Pickard, 2004). The condition, originally described incorrectly as benign, carries the devastating potential for visual loss associated with papilledema and secondary optic atrophy. Diagnosis is made according to the modified Dandy's criteria whereby the patients suffers symptoms of raised intracranial pressure, confirmed by a CSF opening pressure of >25cm H2O (patient lying in lateral decubitus position), with no localizing neurological signs, normal CSF constituents in the presence of a normal computed tomography/magnetic resonance imaging (CT/MRI) brain and no identifiable cause (Corbett and Mehta, 1983). Generally speaking, the condition affects mainly obese females of childbearing

age with an estimated incidence of 3.5/100,000 in females 15 to 44 years of age (Radhakrishnan et al., 1993). The pathophysiology is not completely understood. Experimental studies suggest increased resistance to CSF outflow may be a plausible mechanism (Boulton et al., 1998). The histological features of the brain parenchyma are normal (Wall et al., 1995). Advances in neuroimaging, including gadolinium-enhanced magnetic resonance venography and computed tomography venography, have revealed that transverse cerebral venous sinus stenosis (TSS) is common among patients with IIH (Farb et al., 2003). However, asymptomatic bilateral TSS does exist in patients with a normal intracranial pressure (Kelly et al., 2013). Treatments aim to reduce CSF production and consequently pressure on the optic nerves with lowering of CSF pressure via drainage (a procedure termed lumbar puncture).

#### 3.1.12 Rationale for case group collaborations

In order to compare HMGB1 levels between compartments it is important to examine different disease states. There is no evidence to suggest, nor biological rationale, that the BBB is dysfunctional in IIH and it can therefore reasonably be assumed to be intact and this condition allows the measurement of biomarkers in two separate compartments (CSF and blood). BBB disruption is a pathological feature of neuroinfection and therefore, this condition allows measurement of biomarkers in two separate, but communicating, compartments.

The aims of this study were:

- To determine the concentration of, and relationship between, HMGB1 in paired serum and CSF samples from individuals with headache under investigation for IIH, a non-inflammatory non-infectious CNS control (healthy control substitute).
- To compare HMGB1 concentrations in CSF and blood between noninflammatory conditions in which the BBB is intact (IIH) and in conditions in which it is disrupted (neuroinfection and Rasmussen's encephalitis)

# 3.2 Methods

# 3.2.1 Study Site

The Walton Centre NHS Foundation Trust is a tertiary referral centre for neurology and neurosurgery. The Jefferson Unit day ward contains a clinic room for the purpose of diagnostic and therapeutic lumbar punctures. Patients with a suspected or confirmed diagnosis of IIH are referred to the Jefferson unit for drainage of CSF to relieve headaches associated with raised intracranial pressure.

# 3.2.2 Study population

Otherwise healthy male and female patients (over 16 years of age) with suspected or confirmed idiopathic intracranial hypertension were recruited from the Jefferson dayunit admission register.

The inclusion criteria were:

- Patient was over 16 years of age
- Patient was willing and able to give informed consent
- Patient was scheduled to attend for diagnostic or therapeutic lumbar puncture for the diagnosis or treatment of IIH.
- Patient was healthy with the exception of suspected/confirmed IIH.

The exclusion criteria were:

- Concomitant infection
- Chronic inflammatory/immune-mediated medical condition
- Patient was taking steroids or immunomodulatory therapies
- Patient was unable to provide written consent

# 3.2.3 Sample Size

As this was a pilot study, which has not been conducted previously, an accurate sample size calculation could not be performed. The aim was to recruit 20 patients with suspected/confirmed IIH for initial analysis with a view to increased recruitment if the results showed significant variability in HMGB1 levels. The interim analysis on the

20 patients was discussed with a statistician to determine whether further recruitment was necessary.

# 3.2.4 Ethical approval

Ethical approval for this study was granted by the North West Research Ethics Committee Liverpool East (11/NW/0761). Approval covered the additional collection of blood (maximum 10mls) and CSF (maximum 5mls) from patients undergoing planned (non-emergency) lumbar puncture as part of their clinical care. Approval covered the analysis of novel inflammatory biomarkers.

# 3.2.5 Recruitment

Patients were identified from the list of patients awaiting a day case date for attendance for lumbar puncture at the Jefferson unit. All patients awaiting lumbar puncture for any reason were sent the patient information leaflet (PIL) in the post prior to admission. On the day of admission, the PIL was given to all patients admitted each morning for the procedure. The research physician confirmed that the PIL had been read and understood and sought consent to review the case notes. If the patient was suitable for inclusion in the study consent for inclusion was obtained. This covered the collection of additional blood and CSF from the patient whilst they were undergoing the scheduled lumbar puncture. Patients were included in the analysis if any of the following criteria were met:

- 1. Confirmed IIH (CSF pressure >25mm H20) and attended for drainage.
- 2. Suspected IIH confirmed at that procedure with normal CSF constituents and no other diagnosis confirmed.
- 3. Suspected IIH excluded at that procedure with normal CSF constituents and no other inflammatory or immune mediated diagnosis confirmed.

For criterion 3, if the opening pressure was not elevated, the patient was still included in the study providing review of the investigations (taken as part of the scheduled clinical care investigation) was normal. If the results of the investigations were abnormal or confirmed an alternative diagnosis (for example, multiple sclerosis, a known CNS inflammatory condition) the patients were then excluded from the study and the samples destroyed.

# 3.2.6 Data Collection

The following data was collected from the medical case notes and interview with the patient:

- Demographic details including age, sex, ethnicity, smoking status, alcohol intake
- Symptomatology of presenting complaint requiring lumbar puncture investigation
- Computed tomography/Magnetic resonance imaging brain results if undertaken
- Medical history
- Medication history (type, dose, strength and formulation of therapy)
- Co-morbid illnesses

# 3.2.7 Lumbar puncture and venepuncture procedures

The research physician (Lauren Walker) adequately trained to perform the procedure unsupervised performed all lumbar punctures. Written consent for the procedure was taken following discussion of possible risks and side effects including low-pressure headache, infection, bleeding, temporary or permanent nerve damage (1:10,000-1:100,000).

The patients were positioned in the left lateral decubitus position with the neck and knees fully flexed. Aseptic technique was maintained throughout to minimise the risk of infection. A sterile drape covered the patient and wearing sterile gloves the patient's lumbar spine area was cleansed twice with 5% chlorhexidine solution. The physician palpated to identify the appropriate lumbar vertebrae (L3/L4 or L4/L5). Lignocaine solution (2%, 0.5mls) was infiltrated under the skin and a 20 or 22 gauge spinal needle inserted perpendicular to the skin through the ligamentum flavum until it pierced the dura mater into the subarachnoid space. The stylet from the needle was withdrawn and a CSF column manometer connected to record the opening pressure and confirm that it was elevated. After confirmation, the manometer was removed and the CSF allowed to drip out into collection tubes for analysis and until the CSF

pressure returned to the normal range. The stylet was replaced, the needle removed and pressure applied to the site, which was covered by a sterile dressing.

Immediately following completion of the lumbar puncture, venepuncture was performed to obtain paired serum samples. Both CSF and blood were centrifuged immediately at 2000xg for 20 minutes, the samples were alliquoted and stored at - 80°C until analysis.

# 3.2.8 CNS Infection samples

Paired serum and CSF samples were kindly provided by Dr Griffiths and Dr McGill of the Liverpool Brain Infections Group. Dr Griffiths provided samples from a paediatric Nepalese study investigating suspected Japanese encephalitis in patients with fever, reduced consciousness and confirmed bacterial or viral neuroinfection. Dr McGill provided samples from a Nationwide United Kingdom study (North Wales Research Ethics Committee – West, reference number 11/WA/0218) of patients with confirmed neuroinfection.

# 3.2.9 Rasmussen's encephalitis

Stored, anonymized paired serum and CSF samples obtained from 10 children with confirmed RE prior to 2006 were obtained with ethical approval via the proportionate review service Research Ethics Committee-Greater Manchester West (13/NW/0879). The samples were considered existing holdings under the Human Tissue Act. They were obtained during the clinical care of the patients and stored at -80°C as excess sample following investigation.

# 3.2.10 Serum and CSF HMGB1 quantification by ELISA

A description of the ELISA for serum quantification is provided in 2.2.7. Owing to the relative dilution of CSF compared to serum, CSF samples were analysed according to the highly sensitive procedure (as per the manufacturer's guidelines). The modification includes the use of 50µl of sample (as opposed to 10µl for the standard procedure) added to 50µl of diluent (instead of 100µl in the standard protocol). This produces 1:2 dilution instead of the 1:10 produced for the standard protocol. A total of 50µl of each sample, standard and positive control is then added to the respective wells on the microtitre plate. The rest of the protocol is adhered to as per 2.2.7.

# 3.2.11 Calculation of the CSF:serum albumin quotient

Analysis of serum and CSF albumin for the determination of QALB was kindly undertaken by the Neuroimmunology and CSF Laboratory, University College London Hospitals NHS Trust.

# 3.2.11.1 Statistical analysis

The mean and standard deviation for both compartments in each condition was calculated. Correlation between compartments was determined by the Kendall's tau correlation coefficient (r). Correlation between blood and CSF biomarker levels and clinical variables in patients with neuroinfection was undertaken by Kendall's tau correlation coefficient for continuous variables and Mann-Whitney test for binary variables. The distribution of the data for the CSF patients was non-normal and due to the small sample size non-parametric tests were used for association. The false discovery rate (FDR) (<0.05) was used to correct for multiple testing (Hochberg, 1995).

A multiple imputation technique was attempted to account for the missing data but due to the small sample size and clustering of missing data within the same individuals this was felt to be too inaccurate. Therefore imputation was not performed.

# 3.3 Results

# 3.3.1 Demographics

The demographic details of the healthy control substitute group with suspected/confirmed IIH are detailed in table 3.1. Twenty patients were recruited and analysed, 2 were later excluded due to a subsequent abnormal alternative diagnosis and the samples and results were discarded. 18/20 recruited patients were included in the final analysis. The mean age of the IIH patients was 36.8 years (range 20-58) with a female to male ratio of 10:8.

The demographic details of the patients with neuroinfection are detailed in table 3.2. Sufficient CSF volume for analysis was provided for 19 patients. Paired serum was available for 15/19. The mean age was 15.5 years (range 1.5-68) with a female to male ratio of 10:9. Demographic details for the 10 patients with RE were not available.

Sex	Age (years)	Cigarettes/ day	Alcohol units/ week	Brain imaging (CT/MRI)	Symptoms/Diagnosis	Medical History	Medications
Μ	49	0	2	Normal	Painless visual loss right eye	Nil	Paracetamol 1g QDS
F	29	0	0	Normal	Headache, papilloedema	Nil	Acetozolamide 250mg TDS
Μ	32	0	0.5	Normal	Back pain	Pseudomeningocele following microdiscectomy	Tramadol 50mg PRN
F	26	0	0	Normal	Headache, papilloedema	Nil	Nil
F	44	20	0.5	Normal	Headaches, diplopia, vomiting	Osteoporosis, depression	Citalopram 40mg OD
F	44	0	0	Normal	Headache	Hysterectomy (fibroids)	Nil
Μ	58	0	2	Normal	Headache	Nil	Nil
F	52	0	0.5	Normal	IIH confirmed	IIH 2011	Nil
М	48	0	2	Normal	Headache	Nil	Nil
F	41	0	0	Normal	Headache	Nil	Nil
F	58	0	0	Normal	Headache	Nil	Nil
Μ	32	0	4	Normal	Headache	Nil	Nil
Μ	20	0	0	Normal	IIH confirmed	IIH 2010	Nil
М	19	0	16	Normal	Headache	Nil	Nil
F	44	20	0	Normal	IIH confirmed	benign breast lump, IIH 1998	Nil
F	21	15	0	Normal	IIH confirmed	IIH 2010	Nil

# Table 3.1 Demographics of subjects with suspected or confirmed idiopathic intracranial hypertension

F	41	0	0	Normal	Headache	Nil	Nil
Μ	31	0	20	Normal	Headache, nausea	Hypertension	Lisinopril 20mg OD

Demographics of subjects with idiopathic intracranial hypertension recruited to the "healthy control substitute" arm. M: Male; F: Female; CT; Computed Tomography; MRI: Magnetic Resonance Imaging; IIH: Idiopathic Intracranial Hypertension; g:gram; QDS: Four-times daily; mg:millograms; TDS Three-times daily; PRN: As required; OD:once-daily

# Table 3.2 Demographic characteristics of patients with neuroinfection.

Clinical Diagnosis	Age	Gender	Ethnicity	Fever within 14/7	Seizure	Admission GCS (3-15)
Viral encephailitis	11	Male	SE Asian	Yes	Yes	9
Unknown	2.0	Male	SE Asian	Yes	Yes	10
Encephalitis	9.0	Female	SE Asian	Yes	Yes	10
Transverse myelitis	10.0	Female	SE Asian	Yes	No	14
Japenese Encephalitis	12.0	Female	SE Asian	Yes	Yes	15
Viral encephalitis	5.0	Male	SE Asian	Yes	Yes	15
Dengue	9.0	Female	SE Asian	Yes	Yes	14
Viral encephalitis	6.0	Female	SE Asian	Yes	No	15
Viral encephalitis	12.0	Female	SE Asian	Yes	No	9

Viral encephalitis	6.0	Female	SE Asian	Yes	No	14
Febrile convulsion	1.5	Male	SE Asian	Yes	Yes	7
Viral encephalitis	7.5	Male	SE Asian	Yes	Unknown	15
Pyogenic meningitis	11.0	Female	SE Asian	Yes	No	15
Unknown	8.0	Male	SE Asian	Yes	No	Unknown
Unknown	13.0	Male	SE Asian	Yes	Unknown	Unknown
Unknown	10.0	Male	SE Asian	Yes	Unknown	Unknown
Cryptococcal meningitis	41.0	Male	Caucasian	Yes	No	15
Lymphoma	31.0	Female	Caucasian	Yes	No	Unknown
Pneumococcal meningitis	68.0	Female	Caucasian	Yes	No	8
Pneumococcal meningitis	35.0	Female	Caucasian	Yes	No	15

The study subjects were combined from a paediatric study in South East (SE) Asia and a nationwide study across the United Kingdom of confirmed bacterial meningitis. GCS: Glasgow Coma Score, a numerical assessment of consciousness, minimum 3 and maximum 15.

# **3.3.2** Blood-brain barrier integrity in suspected/confirmed idiopathic intracranial hypertension.

A representative sample (n=7) of paired serum and CSF samples were analysed for the CSF:serum albumin quotient to assess the integrity of the BBB. The BBB was intact in all of the individuals examined; the results are given in table 3.3.

CSF	Result mg/L (RR 90-360)	Serum	Result g/L (RR 34-50)	QALB (CSF/Albumin ratio) (RR <7.2)
01	270	01	45	6.00
02	194	02	45	4.31
03	106	03	41	2.59
04	186	04	39	4.77
05	106	05	46	2.30
06	272	06	44	6.18
07	104	07	42	2.48

# Table 3.3 Blood brain barrier integrity determined by the cerebrospinal fluid:serum albumin ratio

*CSF: Cerebrospinal fluid; RR: Reference range* 

# 3.3.3 HMGB1 in peripheral blood and cerebrospinal fluid

The mean serum concentration ( $\pm$  standard error of the mean) of HMGB1 was: IIH (n=18) 1.43 $\pm$ 0.54; CNS infection (n=15) 25.28 $\pm$ 27.9; and RE (n=10) 1.89 $\pm$ 1.49ng/ml. The mean CSF concentration of HMGB1 was: IIH (n=18) 0.35 $\pm$ 0.22; CNS infection (n=19) 4.48 $\pm$ 6.56; and RE (n=10) 2.24 $\pm$ 2.35 ng/ml. The individual values for CSF and serum HMGB1 are given in table 3.4. The summary statistics for the continuous and categorical variables are described in tables 3.5 and 3.6, respectively.

# 3.3.4 Relationship of HMGB1 between compartments

No correlation was identified between CSF and serum HMGB1 in any of the conditions examined (figure 3.1, a-f).

Idiopathic Intracranial Hypertension		Neuroinfect	ion	Rasmussen's	s encephalitis
CSF	Serum	CSF	Serum	CSF	Serum
HMGB1	HMGB1	HMGB1	HMGB1	HMGB1	HMGB1
ng/ml	ng/ml	ng/ml	ng/ml	ng/ml	ng/ml
0.187	1.005	4.230	8.150	2.577	5.242
0.165	1.299	7.460	43.490	1.090	7.590
0.173	1.417	0.000	69.150	0.655	0.823
0.335	1.292	2.800	13.080	1.254	1.034
0.136	1.321	0.070	97.390	3.800	0.878
0.180	0.924	1.170	17.170	0.710	0.822
0.725	0.961	0.470	6.880	1.025	1.045
0.232	2.418	0.570	40.340	5.135	1.018
0.136	1.454	8.680	41.030	1.134	1.030
0.158	1.402	0.000	0.610	1.529	2.863
0.659	1.807	21.730	3.280		
0.232	1.049	0.000	4.440		
0.291	0.880	3.210	9.960		
0.548	2.507	16.300	14.670		
0.644	2.396	0.520	9.510		
0.563	1.012	24.002			
0.578	1.100	121.031			
		0.048			
		8.532			

Table 3.4 Total values for high mobility group box-1 in paired cerebrospinal fluid and serum

CSF: cerebrospinal fluid; HMGB1: High mobility group box-1

# 3.3.5 Association between serum/CSF HMGB1 and clinical variables

In patients with neuroinfection, a significant positive correlation existed between CSF HMGB1 and CSF white cell count and history of seizure (table 3.7, figure 3.2). Both associations survived multiple testing with FDR.
Variable	n	Mean	SEM	Median	IQR
Blood HMGB1 (ng/ml)	15	25.28	7.21	13.08	33.17
CSF HMGB1 (ng/ml)	19	11.62	6.31	2.80	8.34
CSF white cell count (x10 g/L)	13	453.15	269.60	97.00	195.00
CSF Protein (g/L)	14	44.20	11.31	32.40	63.92
CSF Glucose (mmol/L)	13	41.77	9.33	47.00	44.90
Glasgow coma score	16	12.50	0.76	14.00	5.25
Serum white cell count (x10 g/L)	15	11.73	1.40	11.90	6.47
Serum platelets (x10 g/L)	12	329.83	59.94	265.00	364.80
Serum haemoglobin (g/dl)	15	11.69	0.70	11.30	3.35

Table 3.5 Summary statistics for continuous variables in patients with neuroinfection

Summary statistics for cerebrospinal fluid (CSF) and blood high-mobility group box 1 (HMGB1) in patients with neuroinfection. Results are expressed as mean, standard error of the mean (SEM), median and interquartile range (IQR).

Table 3.6 Summary	v statistics for	categorical	variables in	patients w	ith neuroinfection
		Cutchon loui	Variables III	patients th	

Variable	Categories	n (%)
Seizure*	Yes	7 (44%)
	No	9 (56%)
Abnormal protein**	Yes	8 (57%)
	No	6 (43%)

Summary statistics for binary variables assessed for relationship to HMGB1 in CSF and serum of patients with neuroinfection. \*Status missing for 3 patients \*\*Status missing for 5 patients

Variable	Serum	CSF
	p-value	p-value
CSF white cell count	0.53	0.003
(x10 g/L)		
CSF Protein (g/L)	0.47	0.21
CSF Glucose (mmol/L)	0.79	0.11
Glasgow Coma Score	0.32	0.45
Serum white cell count (x10 g/L)	0.09	0.93
Serum platelets	0.54	0.84
(x10 g/L)		
Serum haemoglobin (g/dL)	0.53	0.96
Seizure (Yes/No)	0.64	0.004

Table 3.7 Associations between clinical variables and serum and cerebrospinal fluidHigh Mobility Group Box-1



Figure 3.1 High-mobility group box 1 (HMGB1) concentrations across serum and cerebrospinal fluid (CSF) compartments in a.) Idiopathic intracranial hypertension (IIH), c.) Neuroinfection and e.) Rasmussen's encephalitis (RE). Box and whisker plots represent mean  $\pm$ SD, n= 18, 15 and 10 respectively. Regression analysis performed using Kendal's Tau correlation coefficient between compartments is not significant in any condition tested, b.) IIH, d.) Neuroinfection, 3.) RE.



Figure 3.2 Cerebrospinal fluid (CSF) High-mobility group box 1 (HMGB1) is significantly associated with CSF white cell count in patients with neuroinfection. Regression analysis was performed by Kendal's Tau correlation coefficient.

#### 3.3.6 Relationship between conditions

In patients with CNS infection, there was a wide range of CSF HMGB1 values. This likely reflects the mixed population of viral and bacterial meningitis. Indeed, the patients with the highest CSF HMGB1 all had confirmed bacterial meningitis. Overall, CSF HMGB1 was not higher in infection versus control; however, isolated analysis of the purely bacterial cases revealed significantly elevated CSF HMGB1 (figure 3.3, A). Regardless of aetiology (viral or bacterial), serum HMGB1 was significantly higher in CNS infection compared to the control group (figure 3.3, A).

In patients with RE, CSF HMGB1 was significantly higher than the control group but this result was driven by a couple of outliers (individual values in table 3.1). There was no statistical difference identified between blood levels (figure 3.4). However, the group numbers were small (n=10).



Figure 3.3 A.) Blood, but not cerebrospinal fluid (CSF), High-mobility group box 1 (HMGB1) was significantly higher in neuroinfection as compared to the healthy-control substitute (IIH). Data presented as mean  $\pm$  standard deviation. Association was determined by Mann Whitney U test, \*\*\*\* p<0.0001.B.) Sub-group analysis of bacterial meningitis only (n=6) revealed significantly higher CSF HMGB1 as compared to control group.



Figure 3.4 Cerebrospinal fluid (CSF), but not blood, High-mobility group box 1 (HMGB1) was significantly higher in Rasmussen's encephalitis (RE) as compared to the control group of patients with idiopathic intracranial hypertension (IIH). Data is presented as mean ± standard deviation. Association was determined by Mann Whitney U test, \*\*\*\* p<0.0001.

#### 3.4 Discussion

This study demonstrates, in conditions involving both normal and abnormally high serum HMGB1 concentrations, that there was no correlation between CSF and serum HMGB1. Therefore, it is not possible to use peripheral blood HMGB1 as a surrogate measure for CSF levels, as there was no predictable relationship demonstrated in this study in any condition. Currently there are no inflammatory biomarkers, detectable in serum or CSF, with proven clinical utility for patients with epilepsy. CSF biomarkers are increasingly considered for other CNS disorders, in particular Alzheimer's disease (AD). The core AD biomarkers (Aβ42, t-tau and p-tau) have shown sensitivity and specificity of 85–95% for AD in discriminating AD dementia from cognitively healthy controls (Landau et al., 2010; Andreasen et al., 2001; Johansson et al., 2011; Hansson et al., 2006). Furthermore, they have been validated and applied, both in combination with and without other clinical indices including clinical data, brain MRI and PET, as an

affirmative diagnostic tool for AD (de Leon et al., 2004; de Leon et al., 2006; Formichi et al., 2006; Schmidt et al., 2015). In AD, CSF biomarker concentrations are acting as a surrogate for inaccessible brain tissue. CSF is mainly produced by the epithelium of the choroid plexus and acts both as a mechanical cushion and as a circulation system for the brain carrying peptides secreted in one region to another and eliminating waste from the brain and spinal cord into the blood circulation (Sakka et al., 2011). Ultimately it is resorbed back into the blood via the venous system. There are small but consistent differences between blood and CSF. CSF has higher concentrations of sodium and chloride, but lower concentrations of potassium, magnesium, bicarbonate, glucose, amino acids and uric acid (Segal, 1993). CSF is also virtually depleted of proteins, reflecting the tight intracellular junctions of the BBB. In addition, the brain has a volume of interstitial fluid, the volume of which is approximately twice that of CSF, about 300 to 400 ml in humans (Segal, 1993). Together the CSF and ISF act as the circulatory network of the brain. Therefore it may be reasonable to assume that the CSF can act as a surrogate for brain tissue. CSF directly contacts nerve tissue and as such, its composition is affected by biochemical changes occurring in the brain (Anoop et al., 2010). However, whether peripheral blood can further act as a surrogate for CSF sampling in some neurological conditions remains to be established. Indeed, in the case of AD, the potential of blood-based AD markers has yet to be further evaluated. Conflicting data regarding blood A $\beta$  levels, which are decreased in the CSF of AD patients, have been reported. One study reported increased plasma A $\beta$  levels in familial AD (Cedazo-Minguez and Winblad, 2010) but others have found that plasma levels of A $\beta$  1–42 and A $\beta$  1–40 are unstable; levels that are elevated, reduced or sometimes even unchanged have been reported (Borroni et al., 2010). Different countries have adopted CSF biomarkers for AD to varying degrees. The National Institute on Aging and the Alzheimer's Association recognise the potential for CSF biomarkers in this disease; they do however state that further research, validation, and standardization (both pre-analytical and analytical) are required (Dubois et al., 2007; McKhann et al., 2011). An important step in determining accuracy with regards to biomarkers concerns the use of accurate analytical methods. In order to reduce variability between laboratories it is important to have established and well-validated cut-off values and rigorous quality control procedures. Accurate quantification of a

particular biomarker in any biofluid requires the use of a method with appropriate analytical validity, for which the Food and Drug Administration provides specific guidance for industry (Food and Drug Administration, 2001b). Specifically, the accuracy, reproducibility and reliability of the method need to have been adequately established. To develop and validate a biomarker test several critical issues must be met. The current gold standard for measurement of total HMGB1 is the commercially available ELISA from Shino-Test Corp., Sagamihara, Japan. The manufacturer's assessment of precision can be found in section 2.1.4. However, validation data for the HMGB1 ELISA, specifically assessing accuracy in CSF measurements, is not available from the manufacturer. As CSF is constituently significantly different to plasma and serum, it is difficult to be confident that the assay is sensitive and specific enough to detect such low protein levels, particularly as are seen in health. Following the highly sensitive protocol, the manufacturer's performance data gives a lower limit of quantification (at which level the assay can be said to be accurate with coefficient of variation <20%) of 0.1 ng/mL. Therefore, particularly in health, the levels of HMGB1 in CSF would be very close to the lower limit of accuracy of the assay.

The present study is the first report to examine HMGB1 levels in both CSF and peripheral blood in IIH, which were found to be within the normal range for health. Recently, speculation about the contribution of some immunologic factors to the pathogenesis of IIH have been cited (Sinclair et al., 2008; Edwards et al., 2013; Altiokka-Uzun et al., 2015). Varying levels of cytokine expression in the CSF in IIH have been shown (Dhungana et al., 2009a; Dhungana et al., 2009b) but this was inconsistent. Some studies have shown that IIH patients show higher CSF IL-17, IL-4, IL-2, IL-10 and IFN- $\gamma$  levels than comparator patients with the inflammatory neurological condition multiple sclerosis (Edwards et al., 2013; Altiokka-Uzun et al., 2015). Similarly to the present study, there were however no correlations between serum and CSF cytokine levels.

This study identifies for the first time, albeit in a small cohort, elevated CSF HMGB1 in patients with Rasmussen's encephalitis when compared to the healthy control substitute, IIH. However, the numbers were small and require further exploration in a larger cohort. In recent years, attention has turned to the role of cytotoxic T cells in the

pathogenesis of RE. Spectral analysis of individual T cells from brain lesions indicates clonal expansion of CD8+ cells, suggesting an antigen-driven CD8+ T cell-mediated autoimmune process (Bauer et al., 2002; Bien et al., 2002). In addition, isoform specific analysis to examine the relative contribution of the acetylation and redox modifications to HMGB1 is required to determine whether the mechanisms of release is necrotic or inflammation driven and whether chemotaxis or cytokine activation are the dominant processes involved.

In this study, CSF HMGB1 was significantly associated with elevated CSF white cell count. CSF pleiocytosis is associated with excessive host immune response in human meningitis and contributes to brain injury (Redzic, 2011; Gekakis et al., 1998; Woodbury and Davenport, 1952; guidelines, 2012). Furthermore, release of inflammatory mediators activates inflammatory cells and promotes vascular permeability, both of which are injurious to tissue, and are associated with poor neurological prognosis (Mustafa et al., 1989; Ohga et al., 1994; Ichiyama et al., 1997). Invading pathogens trigger recognition of PAMPS by Toll-like receptors 2 and 4 (Klein et al., 2008) leading to assembly of the NLRP3 inflammasome. Consequently, large numbers of blood-borne leucocytes are recruited into the leptomeninges. Furthermore, release of HMGB1 from inflammatory and/or necrotic cells is thought to be central to persistent inflammation in pneumococcal meningitis through its chemoattractant function (Hohne et al., 2013). Fully reduced HMGB1 recruits inflammatory cells to compartments undergoing necrosis (Andersson et al., 2014). Injection of HMGB1 into the CSF of mice induces a significant increase in CSF leucocyte counts, an effect blocked by pre-treatment with neutralizing anti-HMGB1 antibodies (Hohne et al., 2013). In a mouse model of pneumococcal meningitis, treatment with ceftriaxone plus HMGB1 antagonist therapy was associated with a significant amelioration of brain pathology compared to ceftriaxone alone (Hohne et al., 2013). The mechanisms responsible for the increases in CSF HMGB1 concentration in neuroinfection are unknown but could reflect intracerebral synthesis, leakage from blood across the disrupted BBB or a combination of these mechanisms.

In summary, the findings from this study comparing the contemporaneous collection of CSF and serum show that no correlation in HMGB1 levels exists between biocompartments in any condition examined, regardless of the integrity of the BBB. It also identifies for the first time that HMGB1 levels are normal in IIH, despite recent reports suggesting an inflammatory pathogenesis. Lastly, elevated serum and CSF HMGB1 was identified in RE, where it may be contributing to the pathogenesis. However, numbers evaluated are small, and replication in a larger sample set with further exploration of the pathological isoforms is required.

# Characterization of HMGB1 Isoforms in Brain and Blood in the Kainic Acid model of Status Epilepticus

#### 4.1 Introduction

Temporal lobe epilepsy (TLE) is a common, frequently difficult-to-control form of epilepsy characterized by complex partial seizures arising from regions within the temporal lobe, usually the hippocampus or amygdala (Chang and Lowenstein, 2003; Bertram, 2009). TLE arises as a consequence of some form of insult to the brain such as neuroinfection, neurotrauma, stroke, prolonged status epilepticus or complex febrile seizure. The process by which normal brain tissue develops a hyper-excitable focus from which spontaneous seizures arise is termed epileptogenesis. TLE is frequently associated with selective neuronal loss in the CA1/CA3 region of the hippocampus and hilus, known as hippocampal sclerosis. This distinct neuropathological pattern occurs along with granule cell dispersion and aberrant mossy fibre sprouting in the dentate gyrus (Berkovic et al., 1991; Thorn, 1997; Jackson et al., 1990; Buckmaster, 2012). In order to develop disease modifying "anti-epileptogenic" drugs able to prevent the development of a drug resistant focus, we need to utilise experimental models of TLE that replicate both the histopathological and behavioural manifestations of the disease.

Currently, the process of epileptogenesis remains incompletely understood. It is unlikely that continual development of anti-seizure drugs, targeting purely the clinical expression of seizures and developed and tested in models of isolated seizures, will yield a successful strategy for disease modification in epilepsy. It is essential that we first unravel the sequence of pathological events that occur within the brain following neurological insult. Furthermore, it has been postulated that "seizures may beget seizures" by aggravating neuronal damage and establishing a negative, deleterious cycle (Ben-Ari et al., 2008). Therefore, there remains a significant unmet clinical need for new drugs that can halt and even prevent the progression of epilepsy following brain insult.

In the last decade, research efforts have focused on the role of inflammation in epileptogenesis. Where it is probable that epilepsy is a multifactorial process, targeting one isolated substrate such as inflammation, may alleviate the downstream cascade of events that leads to disruption of the BBB and development of a hyper-excitable focus.

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#### 4.1.1 The kainic acid model of seizures

A comprehensive description of the KA model can be found in section 1.3.3.1. KA is a potent agonist of the (AMPA)/kainate class of glutamate receptors and is widely used in rodents, either by systemic or intrahippocampal injection, to induce epileptogenesis (Dudek FE, 2006). Activation of KA receptors triggers membrane depolarization and excessive intracellular calcium influx. This in turn leads to neuronal death as a result of mitochondrial dysfunction and generation of reactive oxygen species (Nicholls, 2004; Schinder et al., 1996; Brorson et al., 1994). The KA model induces neuropathological features consistent with human TLE (Ben-Ari and Lagowska, 1978; Ben-Ari et al., 1979). The hippocampus and amygdala are the usual sites of origin of resultant spontaneous electrographic seizures which then propagate to the neocortex (Ben-Ari, 1981; Levesque et al., 2009). Mortality in this model ranges from 5 to 30% (Levesque and Avoli, 2013).

## 4.1.2 Behavioural manifestations of KA-induced seizures and Status Epilepticus

KA (at variable doses depending on administration site and species) induces a period of repeated seizure activity, termed status epilepticus (SE). Definitions of SE in mice can vary in the literature. Acute seizures are described according to the Racine scale (Racine, 1972) and are characterized as:

- freezing and facial clonus (stage 1)
- masticatory movements and head nodding (stage 2)
- wet dog shakes (stage 3)
- forelimb clonus-unilateral or
- bilateral (stage 4) followed by
- rearing and falling (stage 5) (Raedt et al., 2009; Pernot et al., 2011; Mouri et al., 2008).

A generally accepted definition of SE in mice is continuous convulsive seizure activity above Racine stage 3 for a minimum of 30 minutes with incomplete recovery of responsiveness between episodes (Cavalheiro, 1995; Leite et al., 1990; Loscher, 2002). A prolonged duration of seizures (more than 30 minutes) may lead to permanent neuronal damage and synaptic reorganization (Lowenstein et al., 1999). In humans, the spectrum of SE is very wide and indeed classification of SE in humans is a subject of much discussion. Generally speaking, most seizures terminate spontaneously. Closed-circuit video-EEG recordings have demonstrated that the majority of self-limiting seizures in fact last no longer than a few minutes (Theodore et al., 1994; Luders et al., 1993). As a result, the definition has undergone several revisions. The ILAE Task Force on Classification and Terminology defined SE in humans as "a seizure that persists for a sufficient length of time or is repeated frequently enough that recovery between attacks does not occur (1981)." In 1993, the American Epilepsy Society Working Group on Status Epilepticus stipulated a duration of "30 minutes or occurrence of two or more seizures without recovery of consciousness in-between (Brodie, 1990)" however this duration has progressively shrunk in recent years and a minimum length of 5 minutes has since been proposed. The latest definition includes "continuous, generalized, convulsive seizure lasting >5 minutes, or two or more seizures during which the patient does not return to baseline consciousness." Ultimately, failure to recover consciousness between convulsions and persistence of a neurological deficit are considered integral to the diagnosis. Improved level of consciousness would be expected within 20 to 30 minutes of a convulsive seizure.

#### 4.1.3 Scientific rationale for the multiple dosing KA model of TLE

Susceptibility to excitotoxic neuronal injury differs between mouse strains (McKhann et al., 2003; McLin and Steward, 2006; McLin et al., 2006). Administration of a single large dose of KA (by intraperitoneal injection) has the disadvantage that it may cause widespread severe neuronal damage, in excess of the severity observed in humans with TLE, characterized predominantly by cell loss in the hippocampal formation (Sloviter, 1996; Wieser, 2004). Following systemic administration of KA, spontaneous seizures occur 10-30 days after SE (White et al., 2010; Williams et al., 2006; Sharma et al., 2008; Lado, 2006; Drexel et al., 2012; Cherubini et al., 1983; Chauviere et al., 2012). An alternative method is to use focal unilateral low dose injection of KA into the hippocampus creating a restricted focus of damage (Dudek FE, 2006; Ben-Ari, 1985). Cell loss restricted to the CA3 region of the injected hippocampus is observed

7 days after KA injection (Balosso et al., 2005; Ravizza et al., 2006e; Balosso et al., 2008) followed by the appearance of spontaneous epileptic activity occurring reproducibly from approximately 9 days after SE induction for several months (Iori et al., 2013). However, the disadvantage of intrahippocampal injection, particularly in studies examining neuroinflammation, is that it necessitates brain surgery. The animal must be anaesthetized, a section of the skull removed and a guide cannula inserted directly into the brain tissue with KA administered either under anaesthesia or at a later time point. Neuroinflammation occurs as a protective physiological consequence of brain tissue damage. Therefore neurosurgery models present a confounding factor when trying to distinguish the seizure-related effects from surgery-related effects. Examination of post-mortem human brain tissue taken following insertion of depth EEG electrodes shows that procedure-associated immunoreactivity and BBB leakage significantly exceeds the initial area of injury and persists up to at least 330 days following implantation (Liu et al., 2012).

Consequently, for this study of neuroinflammation, the multiple low dosing KA model for rats was adapted to mice (Levesque and Avoli, 2013), whereby animals are given repeated low-dose systemic KA until the onset of convulsive SE. The model was selected for its consistent histological changes which resemble those seen in human medial temporal lobe sclerosis (Hellier et al., 1998). KA passes the BBB very weakly; such low bioavailability means <1% actually reaches the target receptors in the brain (Berger et al., 1986). In multiple dosing, the cumulative dose received by individual mice may vary but the time period during which they undergo repeated seizures is consistent. By comparison to single dose regimens, multiple low dosing regulates seizure activity by titrating dose to the individual seizure threshold. In addition, multiple dosing is known to increase the mortality rate when compared to single dosing in rats. In the rat model, low mortality is coupled with more than 90% of rats developing spontaneous seizures in association with loss of hippocampal neurons, gliosis and mossy fibre sprouting (Hellier et al., 1998). However it must be borne in mind that, compared to intracerebral injection, the bioavailability of KA in the brain may vary between mice receiving different doses of KA systemically, introducing a possible source of bias.

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#### 4.1.4 Scientific rationale for choice of mouse strain

Inbred mouse strains exhibit significant variability in their behavioural response to ictogenic stimuli, including chemoconvulsant agents (Schauwecker, 2002). High seizure-related mortality rates (57%) following a single injection of KA have been shown in C57 and C3H mice, as compared to the 129/SvJ or 129/SvEms mice in which KA is associated with a mortality rate between 0 and 8% (McKhann et al., 2003). The C57BL/6J mouse strain was selected as it is the most common background strain used in transgenic studies; this work then forms a platform for future investigations.

#### 4.1.5 Aims

To examine changes in HMGB1 expression by western blotting (WB) and immunohistochemistry (IHC) in the mouse brain following KA-induced SE in a novel, non-surgical multiple-dosing model.

- 1. To examine the release of the acetylation and redox isoforms of HMGB1 following KA-induced SE.
- 2. To examine the time course of expression of serum HMGB1 following KAinduced SE by quantification of the different molecular forms.

#### 4.2 Methods

#### 4.2.1 Therapeutics

KA (10mg, Abcam, Cambridge, UK.) was dissolved in 2ml distilled water and sonicated for 20 minutes until completely dissolved and stored at -20°C until required.

#### 4.2.2 Experimental animals

Adult male C57BL/6J mice (60 days old, 25-30g body weight, Charles River, Margate, UK) were housed at a constant temperature (23°C) and relative humidity with free access to food and water and a fixed 12-hour light/dark cycle. All efforts were made

to minimize the number of animals used and their suffering. All in vivo work was performed in accordance with the Animals (Scientific Procedures) Act, UK (1986).

#### 4.2.3 Incremental KA

Prior to the induction of seizures, all animals were weighed and separated into individual cages. Seizure intensity was evaluated according to a five-point seizure scale (Racine, 1972; Schauwecker and Steward, 1997), described in table 4.1. Mice were given repeated intraperitoneal (i.p.) injections of 5mg/kg KA at 30-minute intervals until the onset of convulsive SE characterised by the appearance of the first stage-five severity seizure. Generalized seizure activity was monitored and scored at 5-minute intervals thereafter. SE in this model was defined as continuous convulsive seizure activity at or above Racine stage 3 for a minimum of 30 minutes with incomplete recovery of responsiveness between episodes. This is consistent with other models in the literature (Cavalheiro, 1995; Leite et al., 1990; Loscher, 2002).

A major problem with systemic injection of KA is high mortality. Most research groups therefore limit the duration of SE with an anticonvulsant (Loscher, 2002). Seizure activity in this model was terminated after two hours by intramuscular (i.m.) diazepam (10mg/kg). The use of diazepam at this dose and at this time-point does not interfere with subsequent epileptogenesis or the development of associated neuropathology (Ben-Ari et al., 1980; Aroniadou-Anderjaska et al., 2008; Halonen et al., 2001). Sham-treated controls were given i.p. distilled water at 30 minute intervals across a 3 hour timeframe (to mirror KA administration) followed by i.m. diazepam (10mg/kg).

Stage	Description
1	Mouth and facial movements
2	Head nodding
3	Forelimb clonus
4	Rearing
5	Rearing and falling, loss of postural control

Table 4.1 Racine sca	le of seizure	levels in rodents
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Descriptors taken from (Racine, 1972)

## 4.2.4 Euthanasia

Mice were euthanised according to The Humane Killing of Animals under Schedule 1 to the Animals (Scientific Procedures) Act, 1986. KA-treated mice were sacrificed by rising  $CO_2$  at 3, 6, 24, 72 hours and 7 and 14 days following the onset of convulsive SE (figure 4.1). Control mice (n=4) were sacrificed 24 hours following repeated distilled water dosing. A total number of 28 mice were used in this study, 4 in each group.



Figure 4.1 Schedule of dose administration, status epilepticus period and termination with diazepam. Blood and brain collection occurred according to the diagram at 3, 6, 24, 72 hours and 7 and 14 days following the onset of status epilepticus.

## 4.2.5 Blood sample collection

Terminal blood samples were obtained by immediate cardiac puncture and stored on ice. Serum was isolated by centrifugation (5 minutes at 2000xg) and stored at -20°C until analysis.

### 4.2.6 Brain tissue processing

Mouse brains were quickly removed and the right hemisphere rapidly dissected on ice under a stereomicroscope into cortex (frontal and dorsal), hippocampus, cerebellum and brainstem samples. Samples were then snap-frozen in liquid nitrogen and stored at -80°C until used for analysis. Dissected tissue samples from the extracted brains (15mg/sample/region) were homogenised on ice in lysis buffer (Radio-Immunoprecipitation Assay (RIPA) buffer, Sigma Aldrich, UK) containing protease inhibitor cocktail (2µl/ml lysis buffer) and  $\beta$ -mercaptoethanol (0.5µl/ml lysis buffer) for 30s using a TissueRuptor (Qiagen, UK). Homogenates were then centrifuged at 14,000 x g for 10 minutes at 4°C and the supernatants isolated, aliquoted and frozen at -80°C until required for analysis.

#### 4.2.7 Protein Quantification

Total protein amounts were measured using the BCA protein assay method (Sigma-Aldrich, Cambridge, UK). Samples were diluted 1:10 and 1:20 and 9µl of each diluted sample was loaded in duplicate into round-bottom 96-well plates. A 200µl volume of BCA reagent (50:1 BCA:CuSO4) was added to each well and incubated at 37°C for 30 minutes. Absorbance was measured at 570nm and results were fitted to a standard curve generated from known serially diluted concentrations of bovine serum albumin (BSA).

#### 4.2.8 Western Blot Analysis

#### 4.2.8.1 Reagents

Reducing buffer consisting of 30:70 (v/v) NuPAGE<sup>™</sup> Sample Reducing Agent (10X)/ NuPAGE<sup>™</sup> LDS Sample Buffer (4X) (Life Technologies, UK.). Samples were heated at 85°C for 5 minutes to denature the proteins. Running buffer was prepared by 1:20 dilution of NuPAGE<sup>™</sup> MOPS SDS Running Buffer (20X) (Life Technologies, UK.) in distilled water, with NuPAGE<sup>™</sup> antioxidant (1:400) (Life Technologies, UK.) added, as necessary. Transfer buffer was prepared by 1:20 dilution of NuPAGE<sup>™</sup> Transfer Buffer (20X) (Life Technologies, UK.) in 80:20 (v/v) distilled water/methanol, supplemented with 1:1000 NuPAGE<sup>™</sup> antioxidant to enhance transfer of proteins to membranes. Tris-buffered saline (TBS) with Tween-20 (TBST), comprising 10% TBS 10X and 0.1% Tween 20 (Sigma Aldrich, UK), was prepared in distilled water. Chemiluminescence substrate solution consisted of 50:50 (v/v) mixture of Reagent A (luminol) and Reagent B (enhancer) from the Novex<sup>™</sup> ECL HRP Chemiluminescent Substrate Reagent Kit (Life Technologies, UK.)

#### 4.2.8.2 Sample preparation

Samples were defrosted on ice and the volume required from each sample to achieve the requisite amount of total protein ( $10\mu g$ /well, determined by BCA assay, section 4.2.7) was reduced by addition of  $5\mu$ l/sample of reducing agent (Life Technologies, UK). Reduced and denatured samples were then cooled on ice for 10 minutes and vortexed with a whirlmixer.

#### 4.2.8.3 Sample loading and gel electrophoresis

Assembly of the gel electrophoresis running tank (XCell SureLockTM Mini-Cell, Invitrogen, UK) was undertaken according to the manufacturer's manual. The gel cassette (NuPAGE<sup>™</sup> Novex 4-12% Bis-Tris gel, Invitrogen, UK) was inserted into the buffer chamber and the tension wedge locked. The tank was filled with running buffer (1X) supplemented with antioxidant. After the cathode chamber was completely filled with buffer, the external anode chamber was filled with running buffer (1X) without antioxidant, until the buffer reached the level of the wells of the gel. Reduced and denatured samples were loaded onto the gel with a prism protein ladder, which contains 10 proteins that resolve into bands in the range of 2 – 250 kDa (Biorad UK.) The safety lid was closed onto the tank, connected to a PowerPac<sup>™</sup> 300 (Bio-Rad, UK) set at 90V and the gel run for 10 minutes until the lanes had run straight. Thereafter, the voltage was increased to 170V and the gel run for an additional 60 minutes.

#### 4.2.8.4 Transfer of proteins from gel to membrane

After running, samples were transferred onto polyvinylidene difluoride membranes. Gels were placed in the transfer gel sandwich in the following order; white side of the cassette, sponge, filter paper, membrane, gel, filter paper, sponge and finally black side of the cassette sandwich placed on top. Sponges, membranes and filter paper were pre-soaked in 1X transfer buffer. A roller was used to gently roll out air bubbles from the sandwich and the cassette transferred into the module of the transfer tank (Mini Trans-Blot<sup>™</sup> Cell, Bio-Rad, UK). To prevent overheating of the proteins, an ice pack and magnetic stirrer were added to the tank which was filled with 1X transfer buffer. The lid was aligned with the module in the tank and connected to a PowerPac<sup>™</sup> set at 80V and run for 1.5 hours. Once the transfer was complete, the membrane was removed and placed in a plastic tray.

#### 4.2.8.5 Blocking procedures and antibody incubations

Confirmation of the transfer was made using Ponceau S dye (Sigma Aldrich, UK) added to the tray containing the membrane. The membrane was then cut into two parts with a scalpel, one containing the protein region for HMGB1 and one for actin. These were then washed with TBST to remove the Ponceau S dye. The membranes

were blocked with 10% non-fat dry milk prepared in TBST at 4°C overnight under gentle shaking. The membranes were incubated with primary antibodies, either rabbit polyclonal antibody to HMGB1 (Ab18256, 1:5000; Abcam) or mouse monoclonal antibody to actin (1:10,000; Sigma-Aldrich), prepared in 5% skim milk in TBST for one hour at room temperature with gentle shaking. Following primary antibody incubation, the membranes were rinsed with TBST for 20 minutes (TBST discarded and replaced at 5-minute intervals). Secondary antibody incubations, with horseradish peroxidase-conjugated anti-rabbit (HMGB1, 1:10,000; Sigma-Aldrich) and anti-mouse (actin, 1:10,000; Sigma-Aldrich), took place at room temperature for one hour with gentle shaking. After incubation the membranes were again rinsed in TBST for 20 minutes.

#### 4.2.8.6 Chemiluminescence detection of proteins, film exposure and development

Protein bands were visualized by chemiluminescence. The membrane was incubated in pre-mixed chemiluminesence substrate solution for 60 seconds and the excess removed with tissue paper. The membranes were added to the development folder between acetate sheets. In the dark room, photographic film (Carestream<sup>™</sup> Kodak<sup>™</sup> BioMax<sup>™</sup> light film, Sigma Aldrich, UK) was placed on top and developed for the optimum exposure time for each assay. The optimum exposure time had been developed previously and included 10-15 seconds for actin and 30-60 seconds for HMGB1. The films were developed in developer solution (Carestream<sup>™</sup> Kodak<sup>™</sup> autoradiography GBX developer/replenisher, Sigma Aldrich, UK) diluted to 1:4.5 with water, for 5 minutes. The film was then transferred to fixative solution (Carestream<sup>®</sup> Kodak<sup>™</sup> autoradiography GBX fixer/replenisher, Sigma Aldrich, UK) for 5 minutes, also diluted to 1:4.5 with water.

#### 4.2.8.7 Quantification

Films were scanned using a GS-800TM Calibrated Densitometer (Bio-Rad, UK) and captured using Quantity One<sup>™</sup> 1-D Analysis Software (Bio-Rad, UK). Images were quantified using the '1D electrophoresis gel and western blot analysis' within TotalLab Quant tools applications (TotalLab Ltd, UK), with densitometric quantitation of band volumes exported into Microsoft Excel.

#### 4.2.9 Histopathology

Histopathological analysis of the brain tissue samples and cell counting was performed by Dr Emanuele Ricci, Lecturer in Veterinary Pathology, University of Liverpool. The left cerebral hemisphere was removed and post-fixed in 4% Paraformaldehyde (PFA) for 48 hours and rostro-caudally trimmed into serial 1mm thick sections. After routine processing for histology, serial 3µm-thick sections were cut, mounted on polylysinated glass slides (Superfrost Plus<sup>™</sup>; Menzel-Gläser, Braunschweig, Germany), dried overnight at 37°C, and then submitted to haematoxylin-eosin stain.

#### 4.2.9.1 Fluoro-Jade C

Visualisation of Fluoro-Jade C labelled slides was performed according to a previously described protocol (Schmued et al., 2005). Briefly, after de-waxing and hydration, slides were rinsed for 2 minutes in distilled water, and then incubated in 0.06% potassium permanganate solution for 10 minutes. Slides were then transferred for 10 minutes to a 0.0001% solution of Fluoro-Jade C (AG325, Chemicon International, Temecula, CA, USA) dissolved in 0.1% acetic acid vehicle. The proper dilution was accomplished by first making a 0.01% stock solution of the dye in distilled water and then adding 1 ml of the stock solution to 99 ml of 0.1% acetic acid. The working solution was used within 2 hours of preparation. The slides were then rinsed through three changes of distilled water for 1 minute per change. Excess water was drained onto a paper towel, and the slides were then air dried on a slide warmer at 50°C for at least 5 minutes. The air-dried slides were then cleared in xylene for at least 1 minute and then cover-slipped with DPX non-fluorescent mounting media. Fluoro-Jade C labelled slides were visualized using an epifluorescent microscope at the appropriate wavelengths (excitation: 495nm, emission: 521nm, fluorescein/FITC filter).

#### 4.2.9.2 HMGB1, GFAP and IBa1

Microglia were detected using anti-ionized calcium binding adaptor molecule 1 (Iba1), which is a microglia/macrophage-specific calcium-binding protein. GFAP is an intermediate filament protein that is expressed by astrocytes. Consecutive sections were de-waxed in xylene and re-hydrated through serial passages in solutions with

decreasing alcohol concentration (Xylene (neat) x2, 100% ethanol x2, 96% ethanol x1, 85% ethanolx1, 70% ethanolx1, each step was 2 minutes in duration). Endogenous peroxidase activity was quenched with a 30 minute incubation in 20% H2O2 in methanol at room temperature followed by 30 minute incubation with normal swine serum for Peroxidase Anti Peroxidase (PAP) method and normal horse serum (both Vector labs, Peterborough, UK) for ImmPRESS polymer method, in order to prevent non-specific antigen binding. Tissue sections were incubated overnight at 4°C with primary antibody diluted in TBST [rabbit monoclonal anti-HMGB1, 1:250 (Abcam EPR3507); rabbit anti-cow (GFAP), 1:500 (Dako Z0334); goat anti-Iba1 1:1000 (LifeSpan Bioscience LS-B2402)]. After washing with TBST, a 30 minute incubation with swine anti-rabbit (1:100, Dako Z0196) was followed by two washes with TBST and a 30 minute incubation with PAP rabbit (1:250, Dako Z0113) at room temperature. For anti-Iba1 antibody only, anti-goat ImmPRESS polymer was added as a secondary antibody for 30 minutes at room temperature.

#### 4.2.9.3 Image processing and cell count

Cell counting was performed by Dr Emanuele Ricci, Lecturer in Veterinary Pathology, University of Liverpool. For each animal, five non-overlapping microscopic fields consecutively centred on the hilus of the dentate gyrus, CA3, CA2 and CA1 (in duplicate) at 100x magnification were captured with a Nikon Eclipse 80i microscope. After appropriate threshold setting values with ImageJ Software (http://imagej.nih.gov/ij/), all images were processed and analysed using an appropriately designed batch plugin, with generated data exported into an Excel spreadsheet for statistical evaluation. Briefly, after splitting into colour channels, the appropriate threshold was set on the blue channel images and the percentage of the field occupied by GFAP positive astrocytes was evaluated. For HMGB1 positive cells, individual spherical objects were counted using the "analyse particles" function of ImageJ software (circularity level 0.30-1.00, size 355-infinity), whereas for Iba1 marked cells, positive cells were counted with appropriate tested values (circularity 0.00-0.90, size 500-infinity), after exclusion of small processes.

#### 4.2.10 Serum HMGB1 quantification by ELISA

The HMGB1 ELISA method has been described in section 2.2.7.

#### **4.2.11 Liquid chromatography tandem mass spectrometry**

LCMS/MS was performed by Dr Ros Jenkins, Senior Experimental Officer and Dr Daniel Antoine, Lecturer in Pharmacology, University of Liverpool. All chemicals and solvents were of the highest available grade (Sigma-Aldrich, UK). Samples were precleared with 50 µl protein G-Sepharose beads for 1 hour at 4°C. Supernatant HMGB1 was immunoprecipitated overnight with 5 µg rabbit anti-HMGB1 (Abcam; ab18256) for 16 hours at 4°C. The resultant pellet was subjected to SDS-polyacrylamide gel electrophoresis. Protein bands were excised from Coomassie blue-stained gels and destained by incubation with 50% acetonitrile/50mM ammonium bicarbonate followed by vacuum drying. Gel pieces were rehydrated in 50mM ammonium bicarbonate containing 40 ng/ $\mu$ l endoproteinase (GluC) (HMGB1) and incubated for 16 hours at 37°C. Peptides were extracted by incubation with two changes of 60% acetonitrile (ACN)/1% trifluoroacetic acid (TFA) and the resulting supernatants dried. Extracts were desalted using C18 ZipTips according to the manufacturer's instructions and reconstituted in 5% ACN/0.1% TFA. For LC-MS/MS analysis, samples were delivered into a QSTAR Pulsar i hybrid mass spectrometer (Applied Biosystems, Foster City, CA) by automated in-line liquid chromatography (integrated LC-Packings System, 5 mm C18 nano-precolumn, and 75  $\mu$ m × 15 cm C18 PepMap column [Dionex, CA]) via a nano-electrospray source head and 10-µm inner diameter PicoTip (New Objective, Woburn, MA). A gradient from 5% ACN/0.05% TFA (vol/vol) to 48% ACN/0.05% TFA (vol/vol) in 60 min was applied at a flow rate of 300 nl/min. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) spectra were acquired automatically in positive ion mode using information-dependent acquisition (Analyst; Applied Biosystems). Database searching was performed using ProteinPilot 2 (Applied Biosystems) with the latest version of the SwissProt database, with the confidence level set at 80%, and with biological modifications allowed. Analysis yielded more than 75% sequence coverage. Free thiol groups within HMGB1 were alkylated for 90 minutes with 10 mM iodoacetamide at 4°C. Cysteine residues in disulphide bonds were then reduced with 30 mM Dithiothreitol (DTT) at 4°C for 1 hour followed by alkylation of newly exposed thiol groups with 90 mM N-Ethylmaleimide (NEM) at 4°C for 10 min. Samples were subjected to trypsin (Promega, Southampton, UK) or GluC (New England Biolabs, Herts, UK) digestion

according to manufacturer's instructions and desalted using ZipTip C18 pipette tips (Millipore). Characterization of whole protein molecular weights, acetylated lysine residues, or redox modifications on cysteine residues within HMGB1 were determined as described previously by whole protein electrospray ionization or tandem mass spectrometry (Nystrom et al., 2013; Antoine et al., 2012) using either an AB Sciex QTRAP 5500 or an AB Sciex TripleTOF 5600 (Sciex Inc.). Peptide analysis was determined using an AB Sciex QTRAP 5500 equipped with a NanoSpray II source by in-line liquid chromatography using a U3000 HPLC System (Dionex), connected to a 180  $\mu$ m × 20 mm nanoAcquity UPLC C18 trap column and a 75  $\mu$ m × 15 cm nanoAcquity UPLC BEH130 C18 column via reducing unions. A gradient from 0.05% TFA (v/v) to 50% ACN/0.08% TFA (v/v) in 40 min was applied at a flow rate of 200nl/min. The ionspray potential was set to 2200–3500 V, the nebulizer gas to 19, and the interface heater to 150°C.

#### 4.2.12 Statistical analysis

Statistical analysis was undertaken using Graph Pad Prism Software (Graph Pad Prism, San Diego, CA). Pearson's correlation coefficient was calculated to investigate the relationship between quantitative variables of interest, whilst variables were compared between groups using one-way ANOVA with Dunnett's correction for multiple comparisons (normally distributed data) or non-parametric Kruskal Wallis with Dunnett's correction (where data was not normally distributed).

#### 4.3 Results

#### 4.3.1 Seizure threshold and sensitivity to kainic acid

The mean duration of convulsive seizures (minimum Racine stage III and above) was 79.8±7.48 minutes, range 30-120). Sensitivity to KA varied within the group (figure 4.2). There was no relationship between animal weight and the cumulative dose of KA that was required (figure 4.3) or between the cumulative dose of KA and resultant seizure severity (figure 4.4). Seizure severity was determined by the cumulative Racine stage V score (the total number of stage 5 generalised seizures occurring throughout the 120 minute period). The mortality rate of the study was 4%.



Figure 4.2 The cumulative dose of kainic acid (KA) required to achieve status epilepticus (onset of Racine stage V seizures) expressed as percentage of the total number of treated animals (n=24).



Figure 4.3 Relationship between animal weight and total dose of kainic acid (KA) required to induce convulsive seizures following intraperitoneal administration of KA at 5mg/kg every 30 minutes. Weight is expressed as mean  $\pm$  standard error of the mean. Pearson's correlation is illustrated by the solid line, with the corresponding correlation co-efficient reported.



Figure 4.4 Relationship between the cumulative Racine seizure severity score (expressed as mean ± standard error of the mean) and the cumulative dose of kainic acid (KA) required to induce convulsive seizures. Pearson's correlation is illustrated by the solid line, with the corresponding correlation co-efficient reported.

#### 4.3.2 Control mice

No seizures were observed in the control mice. The control group of mice, a small sample (n=4) of vehicle-injected control mice, injected i.p. with distilled water (2ml/kg) 8 times at 30-minute intervals, were sacrificed 24 hours following the initial injection. The degree of animal handling of the vehicle-only control mice was deemed sufficient to account for the stress experienced by the KA-treated mice and there was no significant difference in brain or serum HMGB1 expression in the control group.

#### 4.3.3 Brain expression of HMGB1

#### **4.3.3.1** Total HMGB1 expression in brain homogenates by western blotting

#### 4.3.3.1.1 Hippocampus

At each time point (3, 6, 24, 72 hour, 7 and 14 days) the level of HMGB1 expression normalized to  $\beta$ -actin expression was compared to vehicle-only control. A significant increase in hippocampal HMGB1 from 24 hours following KA-induced SE (control 0.67±0.09 vs 1.70±0.15 (HMGB1/actin ratio, arbitrary units), P ≤ 0.01) persisted to 14 days (1.77±0.32, P ≤ 0.001, figure 4.5). Western blotting quantified the total cellular expression of HMGB1, in this context expression that occurs as a consequence of an inciting event (the KA-induced neuronal damage.)

#### 4.3.3.1.2 Cerebellum

Western blotting revealed a significant increase in cerebellar expression of HMGB1 at 6 hours following the onset of convulsive SE (control  $1.04\pm0.04$  vs  $3.59\pm0.53$ , p<0.001, figure 4.7)



Figure 4.5 Hippocampal expression of high-mobility group box-1 (HMGB1) as determined by western blotting at various time-points up to 14 days following the onset of convulsive seizures. Data is presented as mean  $\pm$  standard error of the mean (n=4/time-point; \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 by one way ANOVA with Dunnett's post-hoc correction for multiple comparisons).



Figure 4.6 Western blot images showing expression of high-mobility group box-1 (HMGB1) in various brain regions from a vehicle-treated control animal and in a further animal at 24 hours following onset of convulsive seizures. Membranes were also stained for 6-actin as a loading control to confirm uniformity. CB: Cerebellum; FC: frontal cortex; DC: dorsal cortex; Hip: hippocampus; BS: brainstem.



Figure 4.7 Expression of high-mobility group box-1 (HMGB1) in the cerebellum as determined by western blotting at various time-points up to 14 days following the onset of convulsive seizures. Data is presented as mean  $\pm$  standard error of the mean (n=4/time-point; \*\*\*p<0.001 by one way ANOVA with Dunnett's post-hoc correction for multiple comparisons).

#### 4.3.3.1.3 Cortex

In the frontal cortex, a trend towards increased HMGB1 expression was seen across time peaking at 24 hours following SE but expression was not significantly different from control at any of the time-points investigated (figure 4.8). In the dorsal cortex, HMGB1 expression peaked at 24 hours following the onset of SE (control 0.45±0.05 vs 0.90±0.71, p<0.05) and returned to baseline by 72 hours (figure 4.9).



Figure 4.8 Expression of high-mobility group box-1 (HMGB1) as determined by western blotting in the frontal cortex at various time-points up to 14 days following the onset of convulsive seizures. Data is presented as mean  $\pm$  standard error of the mean (n=4/time-point.)



Figure 4.9 Expression of high-mobility group box-1 (HMGB1) as determined by western blotting in the dorsal cortex at various time-points up to 14 days following the onset of convulsive seizures. Data is presented as mean  $\pm$  standard error of the mean (n=4/time-point; \*p<0.05 by one way ANOVA with Dunnett's post-hoc correction for multiple comparisons).

#### 4.3.3.1.4 Brainstem

In the brainstem, western blot quantification of HMGB1 expression revealed a significant increase compared to control at 6 hours (control  $0.27\pm0.05$  vs  $1.04\pm0.12$ , p<0.001) and 24 hours (0.95±0.10, p<0.0001) following the onset of convulsive SE (figure 4.10).



Figure 4.10 Brainstem expression of high-mobility group box-1 (HMGB1) as determined by western blotting at various time-points up to 14 days following the onset of convulsive status epilepticus (SE). Data is presented as mean  $\pm$  standard error of the mean (n=4/time-point; \*\*\*p<0.001, \*\*\*\*p<0.001 by one way ANOVA with Dunnett's post-hoc correction for multiple comparisons).

#### 4.3.3.2 Hippocampal histopathology

#### 4.3.3.2.1 Haematoxylin and Eosin Staining and Neuronal cell death by Flourojade C

Starting from 1 hour post SE, minimal neuropathological changes were restricted to the appearance of slightly vesiculous nuclei of astrocytes, associated with mild interstitial oedema at 6 hours post SE. Scattered shrunken, hypereosinophilic and pyknotic neurons ("red dying neurons") were observed at 24 hours post SE, mainly within the CA1 layer, and appeared specifically and intensely marked by Fluoro-Jade C. Occasional necrotic neurons were also scattered within the entorhinal area and thalamic nuclei.

#### 4.3.3.2.2 HMGB1 staining of the hippocampus

Three hours following the onset of SE, HMGB1 positive nuclei staining increased significantly (p < 0.0001, figure 4.11). Within the hippocampi of examined animals, scattered nuclei within the *strati oriens, radiatum* and *lacunosum-moleculare* of the *Cornu Ammonis* and the polymorph layer of the dentate gyrus were specifically labelled. Within the hippocampal hilus, CA3 and CA2, up to 60% of pyramidal neurons were strongly labelled. At six and 24 hours post SE, the number of positive cells and signal intensity dropped back to the level of control animals, before the occurrence of a further intense peak of immunoreactivity at 3 days post SE (figures 4.11 and 4.12 A-D) characterized by prevalent localization of strongly HMGB1 positive cells within CA2 and the neuropil of the adjacent *stratum lucidum*.

#### 4.3.3.2.3 Astrogliosis and Microglial activation

Following KA-induced SE, GFAP positive astrocytes progressively increased in number (predominantly in the *stratum oriens* and *radiatum* of the *Cornu Ammonis*), becoming statistically more numerous at 6 hours (p<0.001) and reaching a peak at 24 hours (also p<0.001) followed by a minimal decrease to levels that were not significantly different from control at 14 days (figure 4.13 A). Cells had a characteristic increase in signal intensity acquiring large GFAP positive cytoplasm with prominent and short cell projections (figure 4.12, E-H).

Similarly, at 24 hours post-SE, Iba1 positive microglia showed a significant increase in number (figure 4.13 B) and with a prominent change in morphology, becoming bipolar or stellate with large strongly labelled cytoplasm and few, shorter and thicker cell processes when compared to the less numerous and finely arborized cells in control animals (figure 4.12, I-L). Morphological changes in Iba-1 positive cells were interpreted as signs of microglial cell activation whereas the increase in cell number within the hippocampus was postulated to represent the local recruitment visible as early as 24 hours after SE. HMGB1 is produced predominantly by astrocytes and not cells of microglial morphology.



Figure 4.11 Hippocampal expression of high mobility group box-1 (HMGB1) by immune staining. Cell count was determined by ImageJ software (http://imagej.nih.gov/ij/). Data is presented as mean ± standard error of the mean (n=4/time-point; \*\*p<0.01, \*\*\*\*p<0.0001 by one way ANOVA with Dunnett's posthoc correction for multiple comparisons).



Figure 4.12 Representative photomicrographs of high mobility group box-1 (HMGB1), Glial fibrillary acidic protein (GFAP) and Ionized calcium binding adaptor molecule 1 (Iba1) immunoreactivity in the hippocampi of kainic acid (KA)-treated C57BL/6 mice (A-L). 40x magnification (A-H) and 200x magnification (I-L).

#### 4.3.3.2.4 Acetylation status

Analysis of the isoforms of HMGB1 present in hippocampal homogenates was undertaken by LCMS/MS. Non-acetylated HMGB1 peaked 3-6 hours following KAinduced seizures, which was followed by a significant rise in the acetylated form at 24 hours (figure 4.14).



Figure 4.13 Quantification of area occupied by GFAP positive astrocyte processes (A) and microglial cells by Iba-1 (B). Data is presented as mean  $\pm$  standard error of the mean (n=4/time-point; \*\*\*\*p<0.0001 by one way ANOVA with Dunnett's post-hoc correction for multiple comparisons).



Figure 4.14 (A) Mass spectrometric characterization of high-mobility group box-1 (HMGB1) acetylation isoforms from mouse hippocampal homogenates following convulsive kainic acid status epilepticus (KA-SE), with data reported as mean fold change from baseline ± standard error of the mean (n = 4/time-point, \*p<0.05, \*\*p<0.01 by Kruskal Wallis test with Dunnett's post-hoc correction for multiple comparisons). (B) Representative spectrum of the tandem mass spectrometric characterization of a peptide (amino acids 180–188) covering the lysine (K) residues within the nuclear localization sequence (NLS) 2 of HMGB1, depicting the hyperacetylated state of HMGB1-NLS2. Acetyl modifications are represented as (ac) on specific lysine residues (K181, K182, K183, and K184).

#### 4.3.3.2.5 Redox status

Significantly elevated expression of both reduced and disulphide HMGB1 isoforms was observed at 6 hours and peaked at 24 hours following KA-induced seizures (figure 4.15). The predominant isoform was the reduced, chemoattractant form,

which would be consistent with leukocyte recruitment to the site of damage for the purpose of repair.



Figure 4.15 Mass spectrometric characterisation of high-mobility group box-1 (HMGB1) redox isoforms from mouse hippocampal homogenates following convulsive kainic acid status epilepticus (KA-SE), with data reported as mean fold change from baseline  $\pm$  standard error of the mean (n=4/time-point, \*p<0.05 by Kruskal Wallis test with Dunnett's post-hoc correction for multiple comparisons).

#### 4.3.4 Serum expression of HMGB1

#### 4.3.4.1 Total HMGB1 quantification by ELISA

Serum HMGB1 rose significantly at 3 hours following the onset of convulsive seizures as compared to vehicle-only control (control  $5.01\pm0.35$  m/ml vs  $16.54\pm0.85$  m/ml, p=0.05) and returned to basal levels by 24 hours ( $4.30\pm2.3$  m/ml). Fourteen days after SE, serum HMGB1 showed a second, significant elevation (control  $5.01\pm0.35$  m/ml vs  $18.03\pm5.73$  m/ml, p<0.05, figure 4.16a).

## 4.3.4.2 Mass spectrometric characterisation of HMGB1 isoforms in mouse serum following convulsive status epilepticus

In KA-treated mice, mass spectrometric characterisation revealed that the early rise in HMGB1 was attributable to the non-acetylated, fully-reduced isoform of HMGB1 (figure 4.16c). Acetylated, disulphide HMGB1 was undetectable until fourteen days
following seizure onset, at which point a significant elevation was seen (figure 4.16b and c).



Figure 4.16 (a) Quantification of HMGB1 by ELISA in serum from control animals and at 3, 6, 24, 72 hr and 7 and 14 days after kainic acid status epilepticus (KA-SE). Results are expressed as the mean ( $\pm$  SEM) concentration in ng/ml (n=4/time-point, \*p<0.05 by one-way ANOVA). (b & c) Mass spectrometric characterization of HMGB1 acetylation and redox isoforms in mouse serum at various time-points up to 14 days following kainic acid status epilepticus. Results are expressed as mean fold change from baseline  $\pm$  standard error of the mean (n = 4/time-point, \*p<0.05, \*\*\*p<0.001, by Kruskal Wallis test with Dunnett's post-hoc correction for multiple comparisons).

#### 4.4 Discussion

In this study, using a novel, multiple i.p. dosing model of KA-induced seizures, no correlation between the total cumulative dose of KA given and the time to onset of Racine Stage V seizure or severity of seizures was identified. Indeed, a wide variation in dose-response was seen across the C57BL/6J mice. The observed variability is consistent with previous reports in the literature that have suggested that C57BL/6J mice show inconsistent responses to systemic KA (Schauwecker and Steward, 1997; Yang et al., 2005b; McKhann et al., 2003; Kurschner et al., 1998). What is more, these and other studies suggest a genetic source of resistance to KA-induced neurotoxicity in the C57BL/6J strain (McKhann et al., 2003; Schauwecker, 2011; Benkovic et al., 2006; De Sarro et al., 2004; McLin et al., 2006). Despite the variation in the occurrence of seizures, significant inflammatory-driven changes evidenced by microglial activation and astrogliosis were seen in the mice, with concomitant necrotic-release of HMGB1 confirming sensitivity of the mice to repeated KA-dosing.

In comparison to the traditional single, high-dose systemic KA model, the model used in this study was labour intensive. Dosing at 30 minute intervals with a wide dose range (from 60 minutes to 3.5 hours) coupled with 5-minute observations of seizure activity throughout the 2-hour SE period meant that a maximum of 4 mice could be observed at any one time. For studies involving euthanasia at 3 and 6 hours following SE onset, the length of the study day ranged from a minimum of 10 to a maximum of 15 hours including time for euthanasia, serum collection, brain dissection and sample processing. As a result, animals were ordered at different times. As the ordering was weight- and gender-based (males, 25-30mg), despite originating from the same supplier, identical parentage cannot be guaranteed. The C57BL/6J strain is the most widely used in-bred strain of laboratory mouse for models of human disease and is mass produced across different study sites. Therefore, the variation in dose sensitivity may be due in part to small differences in the genetic lineage. One possible solution to address this would be to develop an inhouse C57BL/6J strain whereby offspring from the same parents are used for all studies in order to guarantee a constant lineage. However, this homologous 'ideal' does not accurately reflect the human condition. As a matter of fact, there is no

single animal model of epilepsy that can fully represent the disease, particularly given the diverse array of seizure disorders that are subsumed under the umbrella of epilepsy. Ultimately, a degree of variability between rodents was deemed acceptable as it more accurately represents the true clinical situation, wherein epilepsy severity ranges from mild, occasional seizures to daily, uncontrolled generalised convulsions. An alternative solution for future studies would be to increase the number of animals used per time-point in an effort to overcome inherent variability.

Prolonged seizures result in the release of glutamate and other excitatory neurotransmitters. The hippocampus is particularly vulnerable to excitotoxic neuronal injury, having a relative wealth of glutamate receptors. The mechanisms responsible for glutamate-toxicity include intracellular Ca<sup>2+</sup> influx leading to increased Ca<sup>2+</sup>-dependent signalling, and oxidative stress with free radical production and consequent protein and DNA damage (Wang et al., 2005; Rodriguez-Moreno and Sihra, 2004; Carriedo et al., 2000). In the present study, total hippocampal HMGB1 expression rose significantly at 24 hours and persisted from 7 days until 14 days following the onset of SE. It is postulated that cells are damaged/die as a consequence of KA-induced prolonged SE which in turn induces living cells to increase their intracellular pool of HMGB1, which is expressed and detected by western blotting and immunohistochemistry (figure 4.16). Prolonged up-regulation in expression of HMGB1 in the hippocampus, the major site of KAinduced damage in this preclinical epilepsy model, suggests a prolonged, inflammatory-mediated insult with incomplete resolution. This is further supported by reactive astrogliosis and microglial activation visible from 24 hours by IHC analysis. Astrogliosis describes the 'swelling' of astrocytes as they extend projections that then surround damaged and dying neighbouring cells (Sofroniew, 2009) in an effort to limit the degree of neuronal loss and demyelination in response to injury (Sofroniew, 2005; Barres, 2008). Unfortunately, the absence of video EEG in this study means that it was not possible to determine definitively whether the mice underwent epileptogenesis following SE and/or developed spontaneous recurrent seizures. However, the duration of convulsive seizures experienced by the mice in this study is consistent with epileptogenic-inducing brain insults in other epilepsy

models in the literature and therefore it is possible that epileptogenesis was occurring in the mice. The prolonged expression of HMGB1 (24 hours until 14 days) certainly suggests that HMGB1 is overexpressed during the critical epileptogenic period. What remains to be seen is whether it continues to be expressed in the chronic epileptic phase, and a definitive model of spontaneous epileptic seizures is required to confirm this.

Mass spectrometric (MS) characterisation of HMGB1 isoforms in hippocampal homogenates represents a means to identify the amount of HMGB1 that is released from cells into the extracellular milieu, in contrast to western blotting and IHC which confirm changes in cellular expression. MS analysis revealed a shift in the isoforms present, from an early non-acetylated form (3-6 hours) to acetylated HMGB1 at 24 hours. Non-acetylated HMGB1 resides in the nucleus where it functions as an architectural factor to support the structure of chromatin (Stros, 2010). HMGB1 in its acetylated form cannot re-enter the nucleus and thus builds up in the cytosol (Lotze and Tracey, 2005). Acetylation promotes active release from immune cells following sterile inflammation (Lamkanfi et al., 2010; Andersson et al., 2014). Therefore, the shift from non-acetylated to acetylated HMGB1 likely represents delayed immune activation to an initial cellular insult. With respect to function, fully-reduced HMGB1 acts as a chemoattractant (Venereau et al., 2012) whereas disulphide HMGB1 has been shown to be the only form capable of inducing cytokine production (Yang et al., 2012). Therefore, significant elevations in both reduced and disulphide isoforms at 24 hours following SE likely represents initiation of the inflammatory cascade for repair and correlates with the significant rise in cellular expression at 24 hours seen by western blotting.

In primary mouse hippocampal neuron cultures, the disulphide-containing isoform of HMGB1 has been shown to enhance NMDA-induced Ca<sup>2+</sup> increase in neuronal cell bodies in a dose-dependent manner (Balosso et al., 2014). The effect was mediated by TLR4 and blocked by pharmacological inhibition of HMGB1 using the competitive antagonist, Box-A, and a TLR4 selective antagonist. Oxidized HMGB1, considered to be immunologically inert, was incapable of achieving this effect (Balosso et al., 2014).

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Figure 4.13 Graphical representation of the time course of cellular release of high mobility group box-1 (HMGB1) (detected by mass spectrometry) and expression (detected by western blotting and immunohistochemistry) as a consequence of brain insult.

NMDA receptor currents are governed by a balance between phosphorylation (increase) and dephosphorylation (decrease) (Salter, 2009). Phosphorylation is a key form of enhancement of NMDA receptor function achieved by the Src family of protein tyrosine kinases, expressed in neurons of the adult CNS (Cotton and Brugge, 1983). Activation of presynaptic NMDA receptors, following tyrosine phosphorylation of the NMDA receptor subunit 2B (NR2B) (Yu et al., 1997), contributes to excitatory neurotransmission by promoting Ca<sup>2+-</sup>dependent glutamate release (Martin et al., 1991; Suarez et al., 2005). Selective inhibition of Src family tyrosine kinases with 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d] pyrimidine, (PP2) prevented the effect of disulphide HMGB1 on NMDA-induced Ca<sup>2+</sup> influx (Balosso et al., 2014). Importantly, the same signalling is induced by IL-1 $\beta$  via the IL-1R1 receptor (Viviani et al., 2003). Furthermore, both IL-1R1 and TLR4 are contained within the same receptor superfamily and share both a common Toll/interleukin-1 receptor domain and the same adaptor protein (MyD88), both of which are essential for intracellular signalling activation (O'Neill and Bowie, 2007). This data emphasises the potential key role of disulphide HMGB1 as a pathological mediator in seizure, with a specific neuronal action.

In peripheral blood, LCMS/MS analysis confirmed that the early rise seen in HMGB1 was due to the non-acetylated, fully-reduced form. This suggests that early HMGB1 release into the blood may originate from necrotic cells in the brain, in keeping with the purpose of chemotaxis and repair. This is supported by increasing evidence from both experimental models (van Vliet et al., 2007e; Tomkins et al., 2007; Pavlovsky et al., 2005) and human epilepsy (Marchi et al., 2007; Tomkins et al., 2008; van Vliet et al., 2007a) which suggests that disruption of the BBB, permitting spill-over of brain derived mediators into the peripheral system, is a key event in association with seizures. Changes in expression of several potassium and glutamate homeostasis related genes in response to BBB breakdown in rats have been identified (David et al., 2009). In patients with post-traumatic epilepsy, BBB permeability to the MRI contrast medium has been demonstrated co-localised with the presumed epileptic focus (Tomkins et al., 2008; Tomkins et al., 2011). Examination of resected human epileptic brain tissue reveals anatomical abnormalities involving endothelial cells, basal membrane and abnormal tight junctions of the BBB (Cornford, 1999). What remains to be fully clarified is whether this occurs as a prerequisite, or as a consequence, of seizure activity. At present, insufficient clinical evidence exists to confirm that BBB impairment has a definitive role in the generation of epilepsy. Sterile inflammation represents a physiological mechanism to protect and repair brain tissue after injury. A disrupted BBB permits communication between the peripheral and central immune system and hence, elevations in serum HMGB1 in this study may represent spill-over from the necrotic cells within the damaged brain across the disrupted BBB. However, the impact of muscle injury resulting from repetitive seizure activity cannot be excluded in this study and could be responsible for the early rise in necrosis-derived, reduced HMGB1. Unfortunately, due to the relatively large volume of serum required for both ELISA and LCMS/MS in this study (~200µl), insufficient sample was available for analysis of muscle breakdown products, such as creatine kinase, which could have helped to support or exclude this hypothesis. Interestingly, there was a delay in the appearance of the acetylated, disulphide form of HMGB1 in the peripheral blood, which was undetectable until fourteen days following seizure onset at which point a significant elevation was observed. Studies of the KA model of epilepsy have revealed that spontaneous

epileptic activity occurs reproducibly from approximately 9 days after SE induction and persists for several months (lori et al., 2013). However, the absence of video electroencephalography in this study is a limitation, it is not possible to determine whether the mice underwent epileptogenesis and/or developed spontaneous recurrent seizures. Indeed, mice are less likely to develop spontaneous seizures in response to KA compared to rats (McKhann et al., 2003). That being said, the late appearance of the inflammatory form could represent one of two potential sources: 1) Spill over from the CNS due to an ongoing, localised inflammatory reaction, possibly as a result of failure of mechanisms to resolve inflammation or 2) peripherally generated HMGB1 arising in response to the development of spontaneous epileptic seizures.

HMGB1 has recently been revealed to be a critical mediator in the systemic response to brain injury in both experimental and clinical stroke (Liesz et al., 2015). Specifically, HMGB1 signalling via the receptor for glycation end products (RAGE) induces cytokine secretion in the periphery leading to sickness behaviour (weight loss and hypothermia) in mice. The initial release of the non-acetylated and fully reduced form of HMGB1 from necrotic cells post-stroke was followed by delayed appearance of the disulphide form in serum (but not brain) at 24 hours, suggesting maturation to the cytokine-stimulating form occurs following initial necrotic release.

In conclusion, this study shows that multiple, low-dose KA administration in C57BL6 mice induces a brain insult with activation of the innate immune response mediated by the resident microglia and astrocyte population. Concomitant to this, increased expression of HMGB1 occurs in the hippocampus, and other brain regions, from 3- 6 hours following the onset of convulsive seizures. Mass spectrometric analysis of hippocampal tissue confirmed that the early rise resulted from necrotic processes, with the purpose of leukocyte recruitment and repair, which was then followed by an inflammation-driven persistent process at 24 hours. Early release of HMGB1 into the peripheral blood is likely to have resulted from a necrotic process, possibly attributable to spill-over from the central-nervous system. A delayed rise in the inflammatory, disulphide form of HMGB1 occurred 14 days after the KA-SE period, which may coincide with the appearance of spontaneous seizures and provides proof

of principle that HMGB1 represents a pathological isoform, directly connected with the disease process. HMGB1 can induce a persistent pathological inflammation at the site of brain insult, which exceeds the natural homeostasis, turning a once protective repair into a pathologically hyperexcitable neuronal focus. Further investigations to determine whether isoforms of HMGB1 are relevant to provoked seizures, the process of epileptogenesis, spontaneous epileptic seizures or any combination of the three is required.

# Characterization of High-Mobility Group Box 1 Isoforms in Brain and Blood in different Experimental Models of Seizures and Epilepsy

#### 5.1 Introduction

HMGB1 is critically involved in the initiation of the inflammatory cascade in epilepsy and contributes to seizures (Maroso et al., 2010; Balosso et al., 2014; Iori et al., 2013). In addition, it appears to be overexpressed in both brain and blood in relation to prolonged seizure activity (chapter 4). The acetylated disulphide form of HMGB1, originating from inflammatory cells and capable of inducing cytokine release via TLR4, is the one believed to be responsible for its pathological effects in epilepsy (Maroso et al., 2010; Balosso et al., 2014; Iori et al., 2013). These effects can be compounded by the redox state of the extracellular milieu; oxidative stress and cellular injury not only result in the release of cytosolic HMGB1 but also in the generation of reactive oxygen species (ROS) which, in turn, promote the stabilization of HMGB1 in its disulphide form. Thus, there is a vicious cycle that links neuronal injury, the pathological isoform of HMGB1, ROS production, and neuroinflammation. This cascade represents a potential source of biomarkers for CNS disorders in which inflammation is a key pathogenic contributor.

Data from the kainate model (chapter 4) add to the available evidence that HMGB1 is released following prolonged, recurrent seizures as a result of a chemicallyinduced brain insult. In addition, it identified for the first time, delayed expression of inflammatory disulphide HMGB1 occurring in brain (24 hours) and blood (14 days) which may contribute to a persistent, pathological inflammation at the site of insult. What remained unclear from this model was whether the release of HMGB1 occurred as a result of the brain insult, the seizure activity, or both. What is more, particularly in relation to the potentially pathological disulphide isoform, whether inflammatory isoforms of HMGB1 are released following single or recurrent seizures and in normal brain and/or epileptic brain. The disulphide-containing isoform of HMGB1 has been shown to enhance NMDA-induced Ca<sup>2+</sup> increase in neuronal cell bodies which contributes to excitatory neurotransmission (Balosso et al., 2014). The specific mechanism of neuronal action suggests that HMGB1 is involved in the disease process itself and is not simply a marker of recent seizure activity. In order to address these issues, the expression of HMGB1 needed to be examined in models exhibiting the following:

- 1. Brain damage vs. no brain damage
- 2. Chemically induced seizures vs. electrically induced seizures
- 3. Recurrent seizures vs. isolated seizures
- 4. Epileptic brain vs. non-epileptic brain
- 5. Provoked seizures vs. spontaneous epileptic seizures

Different experimental models of seizures and epilepsy were required in order to address whether HMGB1 plays any role in isolated acute seizures (on the background of a normal, healthy brain) or spontaneous, unprovoked seizures arising from an epileptic brain and included the MES test and the pilocarpine-SE model of epilepsy.

# 5.1.1 The maximal electroshock seizure test

The MES test (described in section 1.3.5) has been used for decades for screening anticonvulsant therapies and remains one of the gold standards in early stage AED testing (Rogawski, 2006). It involves non-epileptic animals which are induced to have a single seizure by an electrical impulse. The test involves a stimulus of sufficient intensity to induce maximal tonic extension of the hind limbs (Castel-Branco et al., 2009). The stimulus is approximately 5-10 times higher than the individual seizure threshold of the animals to avoid the bias of daily fluctuations in seizure threshold (Loscher et al., 1991; Piredda et al., 1985; Swinyard and Kupferberg, 1985). The stimulus is applied through transcorneal or transauricular (ear-clip) electrodes. In brief, the stimulus is applied followed by an immediate severe tonic seizure with maximal extension of the anterior and posterior legs and body stiffening. This is the tonic phase, usually lasting 10-15 seconds. After this, clonic seizures commence, characterized by paddling movements of the hind limbs and body shaking. The animal usually returns to an upright position within 20-30 seconds and starts moving around, apparently recovering its normal behaviour (Andre et al., 2002). The test is deemed positive if the animal exhibits tonic extensor seizures with rearward hind limb extension more than 90° from the body which is sustained for more than 3

seconds and occurring within 10 seconds of the stimulation (Castel-Branco et al., 2009). The animals do not go on to develop further spontaneous seizures.

# 5.1.2 The mouse pilocarpine model

The pilocarpine-SE model displays many of the clinical and histopathological manifestations of human mTLE (Covolan and Mello, 2000; Bankstahl and Loscher, 2008; Turski et al., 1987b; Leite et al., 1990) and is described in detail in section 1.3.3.2. Systemic administration of pilocarpine induces a period of SE in rodents, characterized by generalised tonic-clonic convulsions. A generally acceptable definition of SE in rodents is continuous convulsive seizure activity above Racine stage 3 (described in section 4.1.2) for a minimum of 30 minutes with incomplete recovery of responsiveness between episodes (Cavalheiro, 1995; Leite et al., 1990; Loscher, 2002). A latent period of variable duration follows with the appearance of spontaneous recurrent seizures (chronic epilepsy) (Leite et al., 1990; Cavalheiro et al., 1991). Reorganization of hippocampal tissue results, with characteristic mossy fibre sprouting, interneuron loss and ectopic dentate granule cell proliferation, features shared by human mTLE (Wieser, 2004). Pilocarpine exerts its effect via the M1 muscarinic receptor subtype, causing an imbalance between excitatory and inhibitory transmission and an elevation in glutamate levels. Seizures are then maintained by NMDA receptor activation (Nagao et al., 1996; Smolders et al., 1997).

#### 5.1.3 Hypotheses and aims:

The hypotheses for this study were as follows:

- 1. The pathological disulphide isoform of HMGB1 will not be significantly released as a consequence of isolated seizures.
- As a consequence of recurrent spontaneous epileptic seizures, baseline HMGB1 in peripheral blood in epileptic mice exposed to pilocarpine-SE is expected to be greater than healthy controls.

The aims of this study were as follows:

- To characterize HMGB1 acetyl and redox isoform expression in brain and blood following a single seizure in normal, non-epileptic mice using the MES model.
- To characterize HMGB1 expression in brain and serum following both single isolated seizures and spontaneous epileptic seizures using the MES and pilocarpine models.
- 3. To examine the relationship between spontaneous seizure frequency and HMGB1 expression in blood using the pilocarpine epilepsy model.

# 5.2 Methods

# 5.2.1 MES model

The MES test and preparation of serum and brain samples from MES-exposed animals was generously undertaken at our request by colleagues in the Department of Pharmacology & Toxicology at the University of Utah under the direction of Professor Steve White.

#### 5.2.1.1 Mice

Adult male CF1 albino mice (18-25 g) were obtained from Charles River (Portage, Michigan, USA), maintained on an adequate diet (Prolab RMH 3000) and allowed free access to food and water, except during the brief period they were removed from their home cage for MES testing. All mice were housed in plastic cages in specially constructed rooms with controlled humidity, exchange of air, and controlled lighting (12 hours on/off cycle). Mice were housed, fed, and handled in a manner consistent with the recommendations in the National Council Publication, "Guide for the Care and Use of Laboratory Animals". All studies were conducted in accordance with the Institute of Laboratory Resources polices on the humane care of laboratory animals and approved by the University of Utah's Institutional Animal Care and Use Committee (IACUC).

#### 5.2.1.2 MES test procedure

A total of 24 mice were used in this study; 20 mice were subject to MES seizures, with the remaining 4 mice acting as unstimulated controls. MES seizures were

induced with a 50 mA alternating current (frequency = 60 Hz, duration = 0.2 seconds), delivered via silver chloride coated corneal electrodes using an apparatus similar to that originally designed by Woodbury and Davenport (Woodbury and Davenport, 1952). A drop of 0.5% tetracaine hydrochloride in 0.9% saline was applied to the cornea prior to electrode placement. The current delivered was independent of the external resistance and approximately five times that necessary to evoke a tonic hind-limb extension seizure. All stimulated animals in this study experienced a hind-limb extension seizure from which they were allowed to recover.

#### 5.2.1.3 Euthanasia and Sample processing

At each time-point of 1, 4, 8, 16 and 24 hours after the MES-induced seizure, four mice were killed by decapitation and a truncal blood sample was obtained. The whole brains were rapidly dissected onto dry-ice, wrapped in aluminium foil, and stored at -80°C. Serum and brain samples were shipped on dry-ice to the University of Liverpool for analysis, with temperature monitored throughout the transit period.

#### 5.2.1.4 Brain tissue processing and analysis

Frozen brains were defrosted on aluminium foil over ice. The brain was dissected under a microscope. The hippocampus was removed for analysis; the other brain regions (cortex, cerebellum and brainstem) were stored at -80°C. Hippocampal tissue (15mg/sample) was homogenised on ice in lysis buffer (Radio-Immunoprecipitation Assay RIPA-buffer, Sigma Aldrich, UK) containing protease inhibitor cocktail (2µl/ml lysis buffer) and β-mercaptoethanol (0.5µl/ml lysis buffer) following the protocol described in detail in section 4.2.6. Hippocampal homogenates were analysed for the presence of total HMGB1 following the procedures for protein quantification and western blot analysis described in section 4.2.7 and 4.2.8 respectively.

# 5.2.2 The pilocarpine-SE model of epilepsy

The pilocarpine test and preparation of serum and brain samples from pilocarpinetreated animals was kindly undertaken at our request by colleagues at UCB Pharma (Braine l'Alleud, Belgium), under the direction of Dr Rafal Kaminski.

# 5.2.2.1 Mice

Male NMRI mice (Charles River, France) weighing 28–32 g (5–6 weeks old) were used for all experiments in this study. All procedures were carried out according to the Helsinki declaration and the guidelines of the European Community Council directive 86/609/EEC. A local Ethics Committee approved all performed experiments.

# 5.2.2.2 Electroencephalography electrode placement

Mice were surgically implanted with EEG electrodes and allowed to recover for 2 weeks. The video-EEG recordings in mice were performed according to a previously described protocol (Kaminski et al., 2009). Mice were anesthetized with medetomidine hydrochloride (0.5 mg/kg ip, Domitor, Pfizer) and ketamine (50 mg/kg i.p, Imalgene, Rhône- Mérieux) for stereotactic implantation of EEG electrodes. A monopolar depth electrode was implanted into the right hippocampal CA1 region (coordinates vs. bregma: -1.94 mm anteroposterior, -1.0 mm lateral, -1.25 mm depth). Three monopolar cortical electrodes, frontal left (+1.0 mm anteroposterior, -2.0 mm lateral) and occipital left/right: (-4.0 mm anteroposterior,  $\pm 4$  mm lateral) were also positioned on the dura mater. A ground electrode was placed in the left prefrontal bone. EEG activity from hippocampal and cortical electrodes was recorded with a Model 15 Neurodata Amplifier System (Grass Technologies; West Warwick, RI, USA).

#### 5.2.2.3 Induction of Status Epilepticus

Figure 5.1 describes the protocol for seizure induction. After recovery from electrode insertion mice were connected to the video-EEG recording system and pre-treated with N-methylscopolamine bromide (1 mg/kg, i.p.) in order to limit the peripheral cholinergic effects of pilocarpine (Curia et al., 2008; Turski et al., 1987a). Thirty-minutes later, mice were treated with pilocarpine (300mg/kg) via i.p injection. SE appeared within the first hour after pilocarpine injection. SE was interrupted after 2 hours by diazepam injection (10 mg/kg, i.p) and continuous video-EEG monitoring of the mice was undertaken (24 hours per day) for a 7-week period.

#### 5.2.2.4 Video EEG monitoring

A video recording system (Sanyo DSR300) equipped with removable hard drives allowing uninterrupted recording capacity was used to monitor the mice. The recording system consisted of 8 cameras mounted in front of each Plexiglas cage housing individual animals. The cameras were installed inside a sound-proof cabin equipped with 12 hour light–dark cycle. Cameras were capable of infrared recording during the dark phase. Video image was captured at 50 frames per second. Custom made software (UCB Pharma) was used to capture the video signal to digitally synchronize it with the EEG signal for verification of each seizure event (Mazzuferi et al., 2012). Seizures were reviewed at 3× to 6× fast-forward speed and whenever a seizure was detected the video was rewound and reviewed at normal speed to precisely annotate the severity and the duration of each seizure (Mazzuferi et al., 2012).



Figure 5.1 The design of the long-term continuous video-electroencephalography (EEG) monitoring pilocarpine study. Mice were surgically implanted with EEG recording electrodes 14 days before induction of status epilepticus (SE). Pilocarpine (300 mg/kg, intraperitoneal (i.p.)) was injected after 30 minutes baseline EEG recording. SE was limited to 2 hours by diazepam (10 mg/kg, i.p.) injection. Adapted from (Mazzuferi et al., 2012).

# 5.2.2.5 Euthanasia

Mice were sacrificed 28 days after the onset of SE by i.p. injection of 0.3 ml of pentobarbital (Nembutal, 60 mg/ml).

# 5.2.2.6 Control mice

Control NMRI mice (n=4) underwent EEG-electrode insertion, were pre-treated with N-methylscopolamine bromide (1 mg/kg, i.p), and 30 minutes later were given vehicle (1% methyl cellulose (in water) + 0.1% Tween 80 + 0.1% antifoam). This was followed two hours later by diazepam injection (10 mg/kg, i.p.). An additional control group (n=5) of healthy male NMRI mice weighing 28–32 g (5–6 weeks old) was also used for serum analysis.

# 5.2.3 Sample processing

The brain was removed and bisected. The right brain was frozen whole in aluminium foil. The left brain was immersed in 10% formalin and then embedded in paraffin and stored for future use. Blood samples were allowed to coagulate at 4°C for 30 minutes, centrifuged at 1000 x g for 10 minutes at 4°C, and the resulting serum stored at -80°C. Serum and brain samples were shipped on dry-ice to the University of Liverpool for analysis, with temperature monitored throughout the transit period.

# 5.2.4 Brain tissue processing and analysis

The frozen right brain sections were defrosted on aluminium foil over ice. The right brain was dissected under a microscope and the hippocampus removed for analysis. Hippocampal tissue (15mg/sample) was homogenised on ice in lysis buffer (Radio-Immunoprecipitation Assay RIPA-buffer, Sigma Aldrich, UK) containing protease inhibitor cocktail (2µl/ml lysis buffer) and β-mercaptoethanol (0.5µl/ml lysis buffer) following the protocol described in detail in section 4.2.6. Hippocampal homogenates were analysed for the presence of total HMGB1 following the protection quantification and western blot analysis described in section 4.2.7 and 4.2.8 respectively.

# 5.2.5 Statistical analysis

Statistical analysis was undertaken using Graph Pad Prism Software (Graph Pad Prism, San Diego, CA). Single variable comparisons between groups were made using

either one-way ANOVA with Dunnett's correction for multiple comparisons or the non-parametric Kruskal Wallis test with Dunnett's correction. Single variable comparisons between two-groups (treated versus control) were made using the Mann Whitney test. A paired t-test was used to analyse comparisons within the same group of animals.

### 5.3 Results

# 5.3.1 MES

#### 5.3.1.1 Brain total HMGB1 abundance in the hippocampus by western blotting

At each time point (1, 4, 8, 16 and 24 hours) the level of HMGB1 expression normalized to  $\beta$ -actin expression was compared to vehicle-only control. Following a single seizure in the MES model, a trend towards increased hippocampal HMGB1 was observed from 8 hours (control 0.16 ±0.01 vs 0.29 ±0.06 HMGB1/actin ratio, arbitrary units) which reached significance at 24 hours (0.37±0.06, p≤0.0072, figure 5.2).



Figure 5.2 Quantification of western blots for high mobility group box-1 (HMGB1) in mouse hippocampal homogenates from control mice and from mice at various timepoints following maximal electroshock seizures. Data are expressed as mean  $\pm$  standard error of the mean, n=4/timepoint, \*\*p<0.001, by one way ANOVA with Dunnett's post-hoc correction.

Analysis of the isoforms of HMGB1 present in hippocampal homogenates was undertaken by LCMS/MS to allow identification and quantification of the type and amount of HMGB1 that is released from cells. Following an isolated MES-induced seizure, the non-acetylated and reduced forms of HMGB1 peaked significantly at 24 hours (figure 5.3 and 5.4).



Figure 5.3 Mass spectrometric characterization of high mobility group box-1 (HMGB1) acetylation isoforms from mouse hippocampal homogenates at various time-points following maximal electroshock (MES) seizures. Data is presented as fold-increase relative to the baseline. Error bars (means  $\pm$  standard error of the mean, n = 4/timepoint, \*\*p<0.01 by Kruskal Wallis test with Dunnett's post-hoc correction.)



Figure 5.4 Mass spectrometric characterization of high mobility group box-1 (HMGB1) redox isoforms from mouse hippocampal homogenates at various timepoints following maximal electroshock (MES) seizures. Data is presented as foldincrease relative to the baseline. Error bars (means  $\pm$  standard error of the mean, n = 4/timepoint, \*\*\*p<0.01 by Kruskal Wallis test with Dunnett's post-hoc correction.)

### 5.3.1.2 Serum expression of HMGB1

#### 5.3.1.2.1 Total HMGB1 by ELISA

Total serum HMGB1 showed a non-significant peak 4 hours following MES seizures (control 9.22±1.24 vs 19.69±5.64ng/ml, figure 5.5). Isoform analysis and quantification by LCMS/MS confirmed that this early rise, seen both in the MES model and in the KA model (chapter 4), was due to the non-acetylated, fully-reduced form of HMGB1 (figure 5.6, A and B.).



Figure 5.5 Quantification of high mobility group box-1 (HMGB1) by ELISA in serum from control animals and at 1,4,8,16 and 24 hours after maximal electroshock seizures. Results are expressed as the mean ( $\pm$  standard error of the mean) concentration in ng/ml (n=4/time-point).





Figure 5.6 Mass spectrometric characterization of high mobility group box-1 (HMGB1) acetylation (A.) and redox (B.) isoforms in mouse serum following maximal electroshock (MES) seizures . Data is presented as mean  $\pm$  standard error of the mean, n=4/timepoint, \*p<0.05, by Kruskal Wallis test with Dunnett's post-hoc correction.

# 5.3.2 Pilocarpine-SE

### 5.3.2.1 Total HMGB1 abundance in the hippocampus by western blotting

Twenty-eight days following the onset of SE, there was no significant difference in hippocampal HMGB1 abundance, as measured by western blotting, between the healthy controls (mean HMGB1/Actin ratio =  $0.422 \pm 0.09$  arbitrary units, n=4) and those with spontaneous seizures resulting from pilocarpine-SE (0.5251 ± 0.057, n=18, figure 5.7).



Figure 5.7 Hippocampal high mobility group box-1 (HMGB1) expression in the brain of mice treated with pilocarpine-status epilepticus (SE) compared to vehicle-only control. Results are expressed as the mean (± standard error of the mean) ratio of the optical densities of HMGB1 and actin bands, n=4 control and n=18 pilocarpine-SE.

# 5.3.2.2 Serum expression of HMGB1

In pilocarpine-treated epileptic mice, serum total HMGB1 was significantly higher than that of representative control NMRI mice (control 5.32±0.92ng/ml vs 16.59±2.072ng/ml, p=0.0011, figure 5.8).



Figure 5.8 Quantification of high mobility group box-1 (HMGB1) by ELISA in mouse serum from control (n=5) and mice exposed to pilocarpine-status epilepticus and experiencing spontaneous epileptic seizures (n=18). Results are expressed as the mean ( $\pm$  standard error of the mean, \*\*p<0.01 by Mann Whitney test)

#### 5.3.2.3 Relationship between serum HMGB1 and seizure frequency

Serum total HMGB1 was not influenced by (or indeed, did not influence) the frequency of convulsive seizures (Racine stage 3-5) occurring in the 14 days prior to sacrifice (figure 5.9).

#### 5.3.2.4 Relationship between serum HMGB1 and time-since-last seizure

No relationship was identified between serum total HMGB1 concentration and the time since last seizure activity (Racine stage 3-5, Mann Whitney U, p=0.3564). Mice were subcategorized into those that had experienced a convulsive seizure within the preceding 72 hours prior to sacrifice and those that had been seizure-free in that period (figure 5.10). A 72-hour cut-off was chosen solely to ensure adequate numbers of animals in each of the comparator groups.



Figure 5.9 Relationship between total serum concentration of high mobility group box-1 (HMGB1) and total number of convulsive seizures (Racine stage 3-5) experienced across a 14 day period prior to sacrifice. Spearman's rank correlation is illustrated by the solid line, with the corresponding correlation co-efficient reported. The dotted line represents the mean concentration of serum HMGB1 in healthy control mice (n=5, 5.32ng/ml)



Figure 5.10 Box and whisker plots depicting serum high mobility group box-1 (HMGB1) concentrations in mice exposed to pilocarpine-status epilepticus. Each box represents the  $25^{th}$  and  $75^{th}$  percentiles. Lines outside the boxes represent the minimum and maximum limits. Lines inside the box represent the median. Time since last seizure comparison was performed by Mann Whitney test, ns: not-significant, p=0.3564.

# 5.4 Discussion

Both clinical and experimental evidence suggest that HMGB1 is involved in the pathogenesis of seizure disorders (Maroso et al., 2010; Zurolo et al., 2011). What remained unclear was whether up-regulation occurred as a consequence of brain insult, seizures, epileptogenesis or the chronic epileptic state. Together with work undertaken in the KA-model (chapter 4), the present study aimed to identify and clarify whether HMGB1 expression in brain and blood is involved in provoked seizures in normal brain (MES), provoked seizures following brain insult (KA), and/or spontaneous epileptic seizures (pilocarpine-epilepsy) and to provide evidence that HGMB1 expression in blood is not simply a marker of recent seizures.

Following an isolated MES-induced seizure, the non-acetylated form of HMGB1 in brain peaked at 24 hours, consistent with a purely necrotic release process. This is in contrast to the KA-SE model (chapter 4), wherein the non-acetylated form of HMGB1 in brain peaked much earlier, at 3-6 hours following KA-induced seizures. This was then followed by a delayed but significant rise in the acetylated form in brain at 24 hours in the KA model only. This difference in timing likely reflects the extreme difference in models of isolated seizures versus prolonged SE. The MES test is proposed to be a predictive model of generalized tonic-clonic seizures limited to hind-limb extension followed by clonus (Krall et al., 1978). The entirety of the seizure activity lasts less than a minute. In contrast, the excitotoxic glutamate analogue kainate causes widespread neuronal damage to pyramidal cells in the CA3 area of the injected hippocampus (Balosso et al., 2008; Ravizza et al., 2006e).

In the majority of animal models of SE, marked gliosis, axonal sprouting, neuronal cell loss and consequent neurogenesis is seen. However, neuronal damage has been shown to most likely result from the seizure activity itself, rather than as a consequence of the metabolic disturbances that occur alongside SE (Walker et al., 2002). This was revealed by experiments using the GABA antagonist bicuculline to induce SE in adolescent baboons. Bicuculline-induced SE caused hyperpyrexia, severe hypotension, and profound hypoglycaemia in the baboons (Meldrum and Brierley, 1973). However, when the same study was undertaken in paralyzed and mechanically ventilated baboons, to prevent the systemic disturbances, significant neuronal damage was still observed as a consequence of seizures, indicating that prolonged seizure activity is the key pathogenic feature in these models (Meldrum et al., 1973). In addition, the intensity and duration of SE is critical in determining whether an animal will develop spontaneous seizures. Rescue therapy with diazepam within 30 minutes of SE onset limits the degree of neuronal damage and fewer of the animals exhibit spontaneous seizures (White, 2002). Therefore, it is unsurprising that the degree of HMGB1 expression in the brain following a single, brief (<1 minute) seizure was minimal and delayed compared to prolonged, recurrent seizures in the KA model (30 minutes). Furthermore, expression of the inflammatory isoforms of HMGB1 was almost absent in the MES-exposed brain.

HMGB1 in its acetylated form cannot re-enter the nucleus and thus builds up in the cytosol (Bonaldi et al., 2003; Lu et al., 2012; Lu et al., 2014), which is consistent with active inflammatory production. In the KA-SE study (chapter 4), mixed expression of reduced and disulphide HMGB1 peaked at 24 hours. By contrast in this study,

following an isolated seizure the rise was in the reduced form alone. Fully-reduced HMGB1 acts as a chemoattractant (Venereau et al., 2012) whereas disulphide HMGB1 has been shown to be the only form capable of inducing cytokine production (Yang et al., 2012). The relative lack of inflammatory isoforms of HMGB1 in this study of MES seizures showed that HMGB1-mediated inflammatory repair was not initiated as a consequence of a single seizure. The study is limited by a duration of 24 hours and it is therefore possible that inflammatory isoform expression is delayed beyond this time point. However, this is unlikely, given its appearance within 24 hours in the KA-SE model wherein seizure severity is much more severe. MES seizures are not known to induce lasting brain injury and indeed, MES-exposed rodents do not go on to develop spontaneous epileptic seizures (White, 2002). Therefore, the brain results of this study support the notion that HMGB1 inflammatory isoforms are relevant to the epileptogenic process and the subsequent development of epilepsy, and are not merely seizure-related phenomena.

In peripheral blood following single MES-seizure, a non-significant trend towards early release (4 hours) of HMGB1 was seen, and LCMS confirmed the isoform present to be non-acetylated and predominantly in the reduced form. This suggests that early release of HMGB1 following seizure activity is from damaged cells with the capacity to induce chemotaxis for the purpose of repair (Venereau et al., 2012). A later rise in the reduced and to a lesser extent, disulphide isoforms, was seen at 24 hours in the absence of a corresponding elevation in brain. This transient rise occurs likely as a result of monocyte and macrophage release consequent to seizure activity and is indicative of a recent seizure. Indeed, in the KA-SE model, 14 days following the initial SE, a significant rise in acetyl and disulphide HMGB1 was seen, possibly coinciding with the onset of spontaneous epileptic seizures. As discussed in detail in chapter 4, maturation of HMGB1 in peripheral blood, from the necrosis-released reduced form to the cytokine-activating disulphide form occurs following experimental stroke in mice (Liesz et al., 2015). A similar pathology appears to be happening here, with the reduced isoform released early following a seizure (examined in two distinct models, KA and ME) then replaced by the acetylated, disulphide pathological isoforms two weeks after the initial insult.

Taken together, the findings of this study demonstrated that inflammatory isoforms of HMGB1 are not significantly released, in brain or blood, as a consequence of an isolated seizure on the background of normal brain. The study is limited in its time frame to 24 hours following seizures at which point a significant rise in total HMGB1 was seen. It is possible that HMGB1 release, and isoform expression, changes after the first 24 hours and therefore a longer study, up to 72 hours following a single seizure, would be required to exclude this possibility.

In the pilocarpine model of chronic epilepsy, total hippocampal HMGB1 did not differ significantly between mice experiencing recurrent seizures and the shamoperated controls, suggesting that by 28 days following the initial SE, changes in localised HMGB1 expression in brain have resolved. In the KA model, total hippocampal HMGB1 expression remained significantly elevated from 1 to 14 days following the initial SE; therefore resolution of the protective inflammatory reaction driven by HMGB1 likely occurs between 14 and 28 days after brain insult. Taken together, this suggests that HMGB1 is continuously expressed following both initial brain insult and during the critical epileptogenic period, wherein changes are occurring in the hippocampus leading to an excitable focus for future seizures (Wang et al., 2005). Further investigation is required to determine whether the isoforms released differ between healthy animals and those experiencing spontaneous seizures.

In contrast, in the peripheral blood, the epileptic mice experiencing recurrent spontaneous seizures expressed significantly more HMGB1 than control, seizure-free animals. This suggests that ongoing release of HMGB1 may occur as a consequence of recurrent seizure activity, but that the source of the HMGB1 may not be spill over from the CNS and may in fact represent peripheral synthesis. Indeed, it is possible that peripheral immune production of HMGB1 is a driver for seizure activity, crossing the disrupted BBB and entering the brain to aggravate the hyperexcitable focus and induce seizures. This may then contribute to an ongoing cycle involving lowered seizure threshold and consequently, seizures. This is followed by local release of inflammatory mediators including IL-1 $\beta$  and HMGB1. Inflammatory mediators in turn induce intracellular Ca<sup>2+</sup> influx and further seizure activity. Alternatively, it is also

possible that peripheral release of HMGB1 occurs as a consequence of muscle injury resulting from recurrent seizure activity. However, no relationship was demonstrated between serum HMGB1 and either the total number of spontaneous convulsive seizures experienced or the time since last seizure. Furthermore, there is data on file at UCB from accelerometry measurements in these animals which would suggest that they were less active (and moved less far each day) following pilocarpine-treatment than they had done previously, despite experiencing recurrent seizures. Together, this argues against the possibility that HMGB1 is simply a marker of recent seizure activity, released as a consequence of muscle insult. This requires further exploration in humans experiencing both complex partial and generalised seizures, in order to identify the relative contribution of the seizure type. Furthermore, analysis of the muscle injury marker creatine kinase (CK) would help to elucidate whether HMGB1 is released as a consequence of muscular involvement. The white blood cell count (WBC) is known to increase following vigorous physical exercise (McCarthy and Dale, 1988; Tossige-Gomes et al., 2014) and could potentially be the source of peripheral HMGB1.

It is possible that there are other, non-seizure related factors that are involved in the expression of HMGB1 in peripheral blood in chronic epilepsy. Various brain insults are associated both with disruption of the BBB (Abbruscato and Davis, 1999; Betz et al., 1994; Brown and Davis, 2002; Banks, 1999) and a high risk of developing epilepsy (Annegers et al., 1988; Annegers et al., 1998; Jennett, 1975; Burn et al., 1997; Richardson and Dodge, 1954; Sander et al., 1990). Therefore, activation of central immunity in response to brain injury can foreseeably trigger activation of the peripheral immune response, through spill over of inflammatory mediators from the CNS. Indeed, within 24 hours of clinical stroke, stroke patients have been shown to have significantly higher HMGB1 serum concentrations than controls (Muhammad et al., 2008). Similarly, in experimental stroke, elevated HMGB1 serum levels occur 4 hours following occlusion of the middle cerebral artery, without a concomitant rise at the mRNA level (Muhammad et al., 2008). This suggests that, in the early stages, HMGB1 is probably attributable to a spill-over from necrotic neural cells. In contrast, delayed appearance of the disulphide form of HMGB1 in serum (but not brain) has

been shown 24 hours following experimental stroke, suggesting a delayed maturation in the periphery which may well contribute to ongoing release in some individuals. Therefore recurrent seizure activity may be perpetuating release of inflammatory mediators, including HMGB1, which have the demonstrated capacity to provoke and exacerbate seizures (Maroso et al., 2010). Additionally, SE in humans has been associated with excessive muscular activity and relative leucocytosis (Simon, 1985). However, few studies have examined the relationship between single seizure and WBC. In a study involving various seizure types (38 simple partial seizures, 109 complex partial seizures and 91 generalized tonic–clonic seizures) one third of generalized seizures were associated with a significant increase in WBC count. Mean post-seizure sampling time was 21.62 ± 19.33 hours, indicating that in those in which WBC is elevated, the response is delayed. A clear correlation between the length of a seizure and increase in WBC count was seen. Further analysis is required to determine the isoforms present in both brain and blood in the pilocarpine epilepsy model, in particular the disulphide and acetylated isoforms that were shown to be significantly elevated 14 days following KA-SE (Chapter 4). This will help to determine whether release is driven by peripheral immune activation.

In conclusion, the results of this study show that inflammatory isoforms of HMGB1 are not significantly involved within the first 24 hours following isolated MES-induced seizures in mice. In addition, serum, but not brain, total HMGB1 is significantly elevated in chronic epileptic mice experiencing regular spontaneous seizures, the underlying driver for this release remains unclear at present.

# High-Mobility Group Box-1 as a Mechanistic Biomarker in Drug Resistant Epilepsy; Comparing Drug-resistance with Drugresponsiveness.

#### 6.1 Introduction

#### 6.1.1 Inflammation and epilepsy

Substantial experimental and clinical evidence supports activation of inflammatory pathways as aetiopathological in epilepsy disorders (Vezzani and Granata, 2005). Increased microglial and astrocyte inflammatory mediators, including IL-1 $\beta$ , IL-6 and TNF $\alpha$ , trigger a downstream cascade of events leading to activation of adaptive immunity and recruitment of leukocytes into the CNS (Nguyen et al., 2002; Vezzani and Granata, 2005). Inflammation is therefore purported to play a pathological role in seizure generation and the associated neuropathology. Studies have demonstrated that lowering the seizure threshold from the development of hyperexcitability contributes to cell loss, astrogliosis and damage to the blood brain barrier (Vezzani et al., 1999; De Simoni et al., 2000; Vezzani et al., 2000; Vezzani et al., 2005; Balosso et al., 2005; Dube et al., 2005; Heida and Pittman, 2005; Oby and Janigro, 2006; Vezzani and Baram, 2007; Ravizza et al., 2006a).

The role of inflammation during the epileptogenic period in rodents has been well characterized. Activation of the IL-1 $\beta$  system during epileptogenesis is associated with neurodegeneration and BBB breakdown (Ravizza et al., 2008a). Furthermore, in both rats and brain tissue taken at epilepsy surgery from humans with chronic epilepsy, IL-1 $\beta$  and IL-1 receptor type 1 have been shown to be persistently expressed by astrocytes, microglia and neurons, as well as by monocytes and macrophages (Ravizza et al., 2008a). What is more, phenotypic changes in activated microglial cells have been described in chronic epileptic tissue associated with malformations of cortical development (Ravizza et al., 2008a; Boer et al., 2008).

### 6.1.2 Inflammatory mediators in peripheral blood

Whilst many studies have investigated the brain expression of inflammatory mediators following seizure (Eriksson et al., 1999; Vezzani et al., 1999; De Simoni et al., 2000; Plata-Salaman et al., 2000; Turrin and Rivest, 2004; Shinoda et al., 2003; Ravizza and Vezzani, 2006; Gorter et al., 2006), fewer have progressed to examine their subsequent expression in peripheral blood, and fewer still have done so in

humans. Clinical studies have identified that inflammatory cytokines are increased in serum or plasma after seizures (Lehtimaki et al., 2004; Lehtimaki et al., 2007; Lehtimaki et al., 2008; Peltola et al., 2000b). Interleukin-6 (IL-6) and Interleukin-1 Receptor Antagonist (IL-1RA) are elevated in CSF and plasma within 24 hours following both focal seizures and those with secondary generalization with tonicclonic activity (Lehtimaki et al., 2004; Lehtimaki et al., 2010). In patients with established drug refractory TLE, IL-6 is significantly elevated 6 hours post-ictally (Alapirtti et al., 2009). Baseline levels of highly sensitive C-reactive protein (CRP) are significantly higher in patients with drug resistant TLE as compared to healthy controls and secondary generalization of seizures in these patients is associated with a significant elevation of CRP (Alapirtti et al., 2012).

The growing number of studies reporting an association between inflammatory mediators (Aronica and Crino, 2011) and auto-antibodies (Rogers et al., 1994; Levy et al., 2005; Liimatainen et al., 2010) in association with epilepsy also highlights the possibility of an immune-mediated disorder amenable to immunotherapy in some individuals.

#### 6.1.3 Aims

The aims of this study were:

- To compare baseline blood total HMGB1 between patients with wellcontrolled (seizure-free) epilepsy and those with drug-resistant epilepsy and recent seizures.
- 2. To characterize changes in serum HMGB1 relative to timing of seizures in patients with drug refractory epilepsy.
- 3. To characterize acetyl and redox isoforms of HMGB1 in blood in patients with well-controlled and drug-resistant epilepsy

#### 6.2 Methods

Research participants were recruited at the Walton Centre NHS Foundation Trust (n=15) and Tampere University Hospital, Finland (n=50).

# 6.2.1 Study Sites

# 6.2.1.1 Walton Centre NHS Foundation Trust, UK

The Walton Centre has a world-class reputation for epilepsy services, having conducted the largest ever randomised controlled trials of treatment (SANAD, (Marson et al., 2007c)). The comprehensive epilepsy service includes inpatient video electroencephalography (EEG) telemetry wherein patients are admitted for 5-10 days for continuous monitoring of brain wave activity by EEG and video surveillance.

# 6.2.1.2 Tampere University Hospital, Finland

In order to increase the sample size of the study, 50 patients with epilepsy who were admitted to the EEG department in Tampere University Hospital, Finland, were also recruited. The collaborating group was responsible for the design and conduct of the study at their site. Details of the methodology can be found (Alapirtti et al., 2012).

# 6.2.2 UK Study population- Recruitment Process

The study population was recruited from the Walton Centre outpatient services. The patients included those with known drug-resistant epilepsy awaiting elective admission for video EEG telemetry, usually as part of pre-operative assessment for surgical resection. The admissions were a planned part of the patients care.

# 6.2.2.1 UK Patient Identification

Patients were identified by weekly review of all referral forms for inpatient video EEG received by the Neurophysiology department. Referral forms originated from the clinical care team.

#### 6.2.2.2 UK Pre-screening criteria

Patients were selected if the indication for video EEG included the following:

- Chronic drug-resistant focal epilepsy for pre-surgical evaluation
- Chronic focal epilepsy with a change in seizure pattern or frequency
- New diagnosis of epilepsy

Patients were excluded if the indication for video EEG included the following:

 Diagnostic uncertainty where non-epileptic attack disorder (NEAD) is suspected.

Following identification of potentially suitable patients, an invitation letter and a copy of the PIL was included with the patient's standard hospital invitation letter and sent to the patient in advance of the scheduled admission.

The inclusion criteria were:

- Patient willing to take part
- Over 16 years of age
- Written informed consent obtained
- Patient with presumed focal epilepsy
- Scheduled for admission for video EEG telemetry to record seizure activity

The exclusion criteria were:

- Concomitant infections (within 14 days)
- Taking steroids or immunomodulatory therapies
- Any inflammatory or chronic illness
- Unable to obtain written consent

# 6.2.3 Control population

72 healthy volunteers without a history of seizure served as healthy controls. The controls were recruited from the healthy control arm of the BIOPAR study. BIOPAR is an ongoing observational biomarker study collecting longitudinal blood samples from 200 patients with paracetamol overdose. Twenty age and sex-matched patients with established (diagnosis > 12 months prior) well-controlled epilepsy who had been seizure free for longer than 6 months served as epilepsy controls. Epilepsy controls were recruited through the epilepsy outpatient clinic at the Walton Centre.

# 6.2.4 Ethical Approval

Ethical approval for the UK study (Pro-inflammatory Profile of Seizure (PPS) Study: Mapping the Inflammatory Response Following Seizure) was granted by the National Research Ethics Committee North West Haydock Park (10/H1010/55). The Tampere study protocol was approved by the ethics committee of Tampere University Hospital. All patients provided written informed consent.

# 6.2.5 Data collection

The data collected for all patients (Walton and Tampere) was equally comprehensive. The following data was collected from the medical case notes and interview with the patient:

- Demographics (age, sex, ethnicity, smoking status, alcohol consumption)
- Medications and allergies (name/form/dose/duration)
- Medical history, including recent illnesses and prescriptions
- Age at diagnosis
- Seizure frequency, date/time of last seizure, duration seizure-free
- ILAE Epilepsy classification
- Seizure semiology
- Aetiology
- MRI report
- Baseline EEG
- Family history
- Previous febrile seizures

# 6.2.6 Seizure classification:

The studies commenced recruitment prior to the first major overhaul of the epilepsy and seizure classification system by the ILAE. Accordingly, seizures and epileptic
syndromes were classified according to the ILAE 1981 diagnostic criteria (Commission on Classification and Terminology of the International League Against Epilepsy, 1981). Localization of the seizure focus was recorded, and seizures were categorized as simple partial (SPS), complex partial (CPS), primary generalized (PGS) or secondarily generalized tonic clonic seizure (SGTCS).

# 6.2.7 Study Schedule

## 6.2.7.1 Patient Recruitment

At the Walton Centre (UK site), patients were admitted to the ward for video EEG telemetry on a Monday morning. The research physician and/or nurse attended the patient on admission and confirmed prior receipt of the PIL. Following discussion of the study, if the patient consented to inclusion, baseline blood samples were obtained for routine haematology and biochemistry and a 9ml serum sample for biomarker analysis.

# 6.2.7.3 Study specific procedures

The specific investigations undertaken by the participants included:

• Brain magnetic resonance imaging

All patients underwent a diagnostic brain MRI examination on a 1.5 (Tampere) or 3 (Walton) Tesla machine (General Electric Signa HD, Milwaukee, WI, U.S.A.). Evidence of any of the following was classed as abnormal: Hippocampal sclerosis, cortical dysplasia, vascular lesions, gliosis and/or abnormal increased flair signal at the focal site.

• Electroencephalography

All the patients (Walton and Tampere) underwent continuous video-EEG monitoring for electro-clinical characterization of their seizures as part of the routine clinical evaluation for possible epilepsy surgery. Ictal scalp recordings were obtained using synchronous digital video and 24-channel standard bipolar EEG. Electrodes were placed according to the International 10–20 System with additional mastoid and anterior cheek electrodes.

#### 6.2.7.4 Video EEG procedure

At both sites, anti-epileptic drug therapy was reduced and/or omitted and sleep deprivation/photo-stimulation techniques were used in order to increase the likelihood of seizure occurrence. The EEG output was recorded in the Neurophysiology department and was reviewed retrospectively every 24 hours throughout the patient's stay. Any time that the nursing staff or patient reported a seizure the Neurophysiology department was contacted and they then reported on the presence of an EEG correlate. An official report for the duration of the recording was produced for each patient by a Consultant Neurophysiologist.

#### 6.2.7.5 Seizure identification

Seizures were identified by any of the following methods:

- Self-reporting: Patient notified a member of nursing staff who then contacted the neurophysiology department for confirmation.
- Nurse-reported: Nurse in charge of monitoring the video telemetry surveillance screen identified an atypical activity and contacted the neurophysiology department for confirmation.
- Neurophysiology-reported: The neurophysiology technicians contacted the clinical care team to advise of an EEG pattern consistent with seizure.

# 6.2.7.6 Blood sampling

Following seizure activity at the UK site, a 9ml blood sample was obtained immediately and further samples taken 1, 2, 4, 6 and between 8-12 hours following the first (index) seizure. A further sample was obtained upon discharge where possible once the subject had been seizure free for over 12 hours. At the Tampere collaborator site, blood samples were obtained 3, 6, 12, and 24 h after the index seizure.

# 6.2.8 Sample Processing:

Samples were collected in 9ml serum separator tubes. Samples collected during the hours of 08:00 and 18:00 were left upright to coagulate for 10 minutes and then centrifuged at 2000 x g for 20 minutes. Samples collected by the nursing staff out-of-

hours were stored upright in a designated research fridge at 4°C overnight and then processed the following morning. Serum was stored in 500µl aliquots at -80°C until analysis.

#### 6.2.9 Serum HMGB1 quantification by ELISA.

The HMGB1 ELISA method has been described in section 2.2.7.

#### 6.2.10 Serum creatine kinase

Creatine kinase (CK) is an enzyme expressed by various cells types, including predominantly skeletal muscle and myocardium (heart muscle). CK analysis was undertaken by the Clinical Chemistry department at the Royal Liverpool University Hospital.

#### 6.2.11 Statistical Analysis

Statistical analysis was performed in SPSS. Baseline total HMGB1 was compared between 65 patients with drug resistant epilepsy and 20 controls using Kruskal Wallis one-way ANOVA. To test for clinical associations with baseline HMGB1, continuous variables (age, epilepsy duration, seizure frequency, index seizure duration) were tested for association using linear regression, binary variables (MRI, EEG normal/abnormal) using t-test and categorical variables (gender, epilepsy type, seizure type, number of AEDs) using one-way ANOVA. Monthly seizure frequency, index seizure duration and epilepsy duration showed skewed distribution and the data was log transformed to achieve normality. Due to the multiple tests undertaken, the false discovery rate was also calculated for each test. To test for association between clinical variables and the presence of the disulphide-HMGB1 isoform, continuous variables were tested for association using the t-test and categorical variables using Chi-square or Fisher's exact test.

# 6.3 Results:

## 6.3.1 Patient demographics

#### 6.3.1.1 Epilepsy

A total of 67 patients (37 women and 30 men, mean age 34.8 years, range 17-65 years) with DRE were admitted to the video-EEG monitoring units of the Walton Centre NHS Foundation Trust (n=17) and Tampere University Hospital, Finland (n=50) respectively. The patient characteristics are described in table 6-1. Two of the 67 patients had haemolysed baseline samples which were then excluded from the analysis. Summary statistics for the patient characteristics are described in tables 6-2 (continuous variables) and 6-3 (categorical variables).

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						Epile	epsy characteristics	Recorde	ed seizure
	Age (Yrs)	Sex (M/F)	Epilepsy syndrome	Duration (Yrs)	Seizure freq/ month	AEDs (Number)	Brain Imaging	Туре	Duration (secs)
1	36	F	TLE	17	16	2	Hippocampal sclerosis	CPS	72
2	58	F	TLE	42	17.5	3	Hippocampal sclerosis	CPS	61
3	33	F	TLE	22	4	2	Hippocampal sclerosis, left parietal gliosis, left frontal signal change	CPS	67
4	26	Μ	xTLE	22	14	2	Cortical dysplasia in right parietal lobe	CPS	11
5	43	М	TLE	2	1	1	Normal	SGTCS	71
6	22	F	xTLE	3	1	0	Normal	SGTCS	96
7	52	F	xTLE	52	47.5	3	Cortical dysplasia in left frontal lobe and venous anomaly in left parietal lobe	CPS	325
8	39	Μ	TLE	12	15	2	Normal	CPS	13
9	16	F	xTLE	1	35	4	Normal	CPS	17
10	26	Μ	xTLE	9	200	3	Vascular lesion in the right temporoparietal lobes	SPS	50
11	45	F	IGE	32	1.5	4	Vascular lesion left frontotempoparietal lobe	PGS	70
12	30	Μ	TLE	25	2.5	3	Hippocampal sclerosis and hamartoma	CPS	60
13	41	F	xTLE	31	31	2	Normal	CPS	24
14	52	Μ	TLE	52	5	3	Hippocampal sclerosis and signal change in left frontal lobe	CPS	105

# Table 6.1 Clinical characteristics of patients with drug-resistant epilepsy

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15	31	F	xTLE	31	75	3	Normal	CPS	192
16	58	F	TLE	32	9.5	3	Normal	CPS	83
17	22	F	xTLE	17	60	4	Cortical dysplasia in right frontal lobe	CPS	14
18	28	Μ	TLE	16	1	2	Hippocampal sclerosis	SGTCS	62
19	57	F	TLE	56	3	1	Hippocampal sclerosis	CPS	115
20	20	F	TLE	14	26	3	Cortical dysplasia in right and left temporo-parietal lobes	SPS	6
21	37	Μ	xTLE	37	6	3	Normal	SGTCS	101
22	21	Μ	xTLE	4	22.5	3	Signal change in right frontal lobe	CPS	12
23	23	Μ	xTLE	23	30	3	Normal	CPS	20
24	33	Μ	xTLE	15	5	1	Normal	CPS	20
25	32	Μ	TLE	7	0.5	1	Hippocampal sclerosis	CPS	182
26	23	F	xTLE		25	1	Normal	CPS	5
27	45	Μ	xTLE	5	75	3	Signal change in the right gyrus cingulare	SPS	60
28	38	Μ	TLE	4	4	4	Gliosis in right temporal lobe	CPS	142
29	27	F	IGE	17	0.5	2	Normal	PGS	60
30	35	Μ	TLE	35	6	2	Normal	CPS	67
31	18	F	xTLE	7	15	2	Cortical dysplasia in the left frontal lobe	SPS	26
32	44	F	TLE	26	0.5	2	Hippocampal sclerosis	SGTCS	129
33	42	Μ	TLE	39	10	2	Hippocampal sclerosis	CPS	414
34	32	Μ	xTLE	25	60	2	Left vascular lesion and bilateral cortical dysplasia	CPS	25

35	49	F	xTLE	31	0.5	3	Normal	SPS	5
36	47	F	XTLE	36	240	2	Right cortical dysplasia	CPS	34
37	20	F	xTLE	4	0.5	2	Normal	CPS	41
38	27	F	IGE	18	1.5	1	Normal	PGS	65
39	39	F	TLE	19	22	2	Hippocampal sclerosis	CPS	34
40	31	F	xTLE	5	240	3	Right slight hemimegaloencephalia	CPS	26
41	28	F	TLE	24	8	2	Hippocampal sclerosis	CPS	90
42	47	F	TLE	4.5	3	3	Left parietal gliosis after meningeoma operation (8/01) and	CPS	7235
							left temporo-mesial atrophy		
43	25	М	xTLE	13	60	3	Vascular lesion in right frontotemboral lobe	CPS	135
44	45	Μ	XTLE	44	70	4	Normal	CPS	5
45	46	Μ	TLE	33	5	1	Cortical dysplasia in left occipital lobe	CPS	77
46	17	Μ	XTLE	15	28	3	Normal	SGTCS	68
47	43	Μ	TLE	24	30	2	Cortical dysplasia in left temporal lobe	CPS	39
48	27	F	IGE	6	4	1	Normal	PGS	67
49	31	F	TLE	19	3	2	Hippocampal sclerosis	CPS	118
50	32	Μ	TLE	9	4	2	Signal change in left amygdala	CPS	78
51	27	F	TLE	8	28	3	Normal	SGTCS	n/a
52	20	F	IGE	3	4	2	Normal	CPS	n/a
53	30	F	TLE	18	2	3	Hippocampal sclerosis	CPS	n/a

54	28	F	TLE	2	4	2	Normal	SPS	n/a
55	46	Μ	TLE	25	28	3	Normal	SGTCS	n/a
56	26	F	TLE	16	4	4	Normal	CPS	n/a
57	53	Μ	TLE	28	12	3	Hippocampal sclerosis	CPS	n/a
58	39	Μ	IGE	14	6	4	Normal	SGTCS	n/a
59	32	Μ	TLE	9	4	3	Hippocampal sclerosis	CPS	n/a
60	65	F	TLE	40	1	2	Normal	CPS	n/a
61	31	Μ	IGE	12	12	3	Normal	CPS	n/a
62	60	F	TLE	13	4	4	Normal	CPS	n/a
63	23	F	xTLE	14	8	2	Normal	SGTCS	n/a
64	22	F	xTLE	22	84	2	Normal	SGTCS	n/a
65	46	М	xTLE	8	12	3	Left hippocampal atrophy	CPS	n/a

M: Male; F: Female; TLE: Temporal Lobe Epilepsy; xTLE: Extra-temporal lobe epilepsy; IGE: Idiopathic Generalised Epilepsy; CPS: Complex Partial Seizure; SGTCS: Secondarily Generalised Tonic Clonic Seizure; PGS: Primary Generalised Seizure; SPS: Simple Partial Seizure; n/a: information not captured

Variable	n	Mean	SD	Median	IQR
Age	65	35.18	11.96	32	29.00
Epilepsy Duration	65	19.53	13.70	17	27.00
Seizure Frequency	65	27.31	48.65	9.50	24.00
Index Seizure Duration	52	213.38	995.62	66	71.50
Baseline HMGB1	65	8.70	3.81	8.94	2.91
Peak post-seizure HMGB1	59	10.56	4.88	9.85	2.46

Table 6.2 Summary statistics for continuous clinical variables examined in patients with drug-resistant epilepsy.

SD: standard deviation; IQR: Interquartile range

Table 6.3 Summary statistics for categorical clinical variables examined in patients with drug-resistant epilepsy.

Variable	Categories	n(%)
Gender	Male	29 (45%)
	Female	36 (55%)
Epilepsy Type	IGE	7 (11%)
	TLE	32 (49%)
	xTLE	26 (40%)
Index Seizure Type	PGS	4 (6%)
	CPS	44 (68%)
	SPS	6 (9%)
	SGTCS	11 (17%)
High highly-sensitive C- reactive protein (CRP)*	Yes	33 (63%)
	No	19 (37%)
Redox Disulphide	Yes	24 (37%)
	No	41 (63%)
MRI	Normal	30 (46%)
	Abnormal	35 (54%)
EEG	Normal	4 (6%)
	Abnormal	61 (94%)
Number of AEDs	0	1 (1%)
	1	8 (12%)
	2	24 (37%)
	3	24 (37%)
	>3	8 (12%)

TLE: Temporal Lobe Epilepsy; xTLE: Extra-temporal lobe epilepsy; IGE: Idiopathic Generalised Epilepsy; CPS: Complex Partial Seizure; SGTCS: Secondarily Generalised Tonic Clonic Seizure; PGS: Primary Generalised Seizure; SPS: Simple Partial Seizure; MRI: Magnetic Resonance Imaging; EEG: Electroencephalography; AEDs: Antiepileptic drugs.\*Status for 15 patients missing (UK study)

#### 6.3.1.2 Controls

Seventy-two healthy volunteers without history of seizure served as healthy controls (37 women and 35 men, mean age 34.1 years, range 19-66 years). Twentypatients with established (diagnosis > 12 months prior) of well-controlled epilepsy (9:11 symptomatic:idiopathic, 12:8 monotherapy:dual therapy) who had been seizure free for longer than 6 months served as epilepsy controls (12 women and 8 men, mean age 33 years, range 18-59 years), described in table 6.4. Comparative characteristics of the patient groups are described in table 6.5.

	Age (years)	Sex	Epilepsy Syndrome	Duration (years)	Duration seizure- free (months)	AEDs (number)
1	26	Μ	TLE	11	24	1
2	73	F	IGE	18	24	1
3	19	Μ	JME	2	12	1
4	45	F	IGE	26	11	2
5	39	F	TLE	2	11	2
6	19	F	TLE	9	6	2
7	28	Μ	xTLE	10	6	1
8	24	М	IGE	5	14	2
9	42	F	xTLE	34	13	1
10	24	F	xTLE	12	20	1
11	27	Μ	JA	11	24	1
12	32	М	IGE	4	6	1
13	25	F	xTLE	22	24	2
14	59	Μ	IGE	2	6	1
15	21	F	IGE	2	15	2
16	42	F	TLE	8	10	1
17	24	F	TLE	11	14	1
18	22	F	IGE	15	84	1
19	48	Μ	xTLE	3	36	1
20	21	F	IGE	6	18	1

Table 6.4 Clinical characteristics of patients with well-controlled epilepsy who have been seizure-free for longer than 6 months.

*M:* Male; F: Female; TLE: Temporal Lobe Epilepsy; IGE: Idiopathic Generalised Epilepsy; JME: Juvenile Myoclonic Epilepsy; xTLE: Extra-temporal lobe epilepsy; JA: Juvenile Absence

	Healthy controls	Epilepsy seizure-free (min. >6m)	Drug-resistant epilepsy
Number of Patients	72	20	65
Age, years (range)	34.1 (18-49)	33 (19-73)	35.1 (17-65)
Male/Female	35/37	8/12	29/36
Epilepsy Type (symptomatic/Idiopathic)	n/a	9/11	58/7
Mean duration epilepsy, years (range)	n/a	10.7 (2-34)	19.5 (1-56)
Anti-epileptic therapy (mono/polytherapy) (mono/dual/triple/Quad)	0	12/8 12/8/0/0	8/57 8/24/25/8
Mean monthly seizure frequency (range)	0	0.004 (0-0.2)	27.31 (0.5-240)
Serum HMGB1 ng/ml, mean ± s.e.m	1.11±0.07	1.25±0.15	8.70 ±0.47

Table 6.5 Comparative characteristics of the patient groups.

HMGB1: High mobility group box-1

# 6.3.2 Measurement of HMGB1

# 6.3.2.1 Baseline

Compared to both healthy controls (1.11 $\pm$ 0.07ng/ml, p<0.0001) and those with wellcontrolled epilepsy (1.25 $\pm$ 0.15ng/ml, p<0.0001), mean baseline total HMGB1 was significantly higher in patients with drug-resistant epilepsy (8.70  $\pm$ 0.47ng/ml) (figure 6.1).

# 6.3.2.2 Correlation with clinical variables

An association between baseline HMGB1 and duration of the index seizure was identified (table 6.6); however this association did not withstand correction for multiple testing with FDR. Abnormal brain MRI correlated with high baseline HMGB1 (p=0.007, table 6.6, figure 6.2).



Figure 6.1 Quantification of total serum high mobility group box-1 (HMGB1) by ELISA in healthy controls and patients with well-controlled seizure-free epilepsy and drug resistant epilepsy. Results are expressed as the mean ( $\pm$  standard error of the mea, n=72, 20 and 65 respectively; \*\*\*\*p<0.0001 by Kruskal Wallis one way ANOVA.)

Table 6.6 Tests of association between clinical variables and baseline high mobility group box-1.

Variable	Ν	p-value
Age	65	0.736
Gender	65	0.829
Square Root Epilepsy Duration	64	0.219
Log Seizure Frequency	65	0.492
Log Index Seizure Duration	50	0.028
Epilepsy Type	65	0.866
MRI Abnormal	65	0.009
EEG Abnormal	65	0.574
Number of AEDs	65	0.036

Continuous variables (age, epilepsy duration, seizure frequency, index seizure duration) were tested for association using linear regression, binary variables (MRI, EEG normal/abnormal) using t-test and categorical variables (gender, epilepsy type, seizure type, number of AEDs) using one-way ANOVA.



Figure 6.2 In patients with drug-resistant epilepsy, abnormal brain magnetic resonance imaging (MRI) was significantly correlated with elevated baseline high mobility group box-1 (HMGB1). Error bars (means ± standard error of the mean, n=65); \*\*p< 0.01 by t-test.

# 6.3.2.3 Post-translational modifications of HMGB1

Clear separation of isoform expression was visible between the patient groups depending on whether they were drug-responsive or not. Thus, acetylated HMGB1 was observed in drug-resistant epilepsy patients alone; these patients could be further sub-

stratified on the basis of the disulphide inflammatory form (figure 6.3). There was no significant association between the presence of disulphide HMGB1 and any of the clinical variables tested (table 6.7.)



Figure 6.3 The acetylated form of high mobility group box-1 (HMGB1) was observed in drug resistant patients alone, and these individuals could be further sub-stratified on the basis of redox state of HMGB1. Disulphide inflammatory HMGB1 was detectable in a sub-cohort of patients with drug-resistant epilepsy with significantly higher baseline HMGB1. Data is presented as the mean ( $\pm$  standard error of the mean, n=72, 20 and 65 for controls, epilepsy seizure-free and drug resistant epilepsy respectively; \*\*p<0.01, \*\*\*\*p<0.0001 by Kruskal Wallis one way ANOVA.)

Variable	Ν	p-value
Age	65	0.801
Gender	65	0.900
Square Root Epilepsy	64	0.733
Duration		
Log Seizure Frequency	65	0.606
Log Index Seizure Duration	52	0.606
Epilepsy Type	65	0.079
MRI Abnormal	65	0.183
EEG Abnormal	65	1.00
Number of AEDs	65	0.713

Table 6.7 Testing association with disulphide high mobility group box-1.

*Continuous variables tested with t-test and categorical variables, Chi-square or Fisher's exact test used.* 

#### 6.3.2.4 Peak Post-Seizure

59/65 patients experienced a seizure during the video-EEG inpatient admission. The baseline and peak-post-seizure samples are described in table 6.8. Peak post-seizure HMGB1 was significantly elevated compared to baseline admission HMGB1 (figure 6.5).

An association between peak post-seizure HMGB1 and age was identified (table 6.9); however this did not withstand correction for multiple testing with FDR.

#### 6.3.2.5 Post-seizure profiles

Post-seizure serum samples were collected from patients that experienced an inpatient seizure at different time points (range 0.5-24 hours). Individual profile plots for total HMGB1 at baseline and following seizure are shown in figure 6.6.



Figure 6.4 Quantification of high mobility group box-1 (HMGB1) by ELISA in human sera at baseline and the peak post-seizure sample. Peak post seizure sample timing varies between patients (0.5-24 hours). Results are expressed as the mean ( $\pm$  standard error of the mean, \*p<0.01 by paired two-tailed t-test)

#### 6.3.2.6 Creatine kinase

Postictal creatine kinase (CK) rises are postulated to be characteristic of generalized tonic clonic seizures, resulting from intense muscle contraction and muscle ischeamia (Wyllie et al., 1985). The normal range for CK in males is 40-320U/L and 25-200U/L in females. Baseline and peak HMGB1 post-seizure samples were analysed for the presence of CK in 54 patients who experienced a seizure (table 6.8). CK was elevated above the normal range in only 5 subjects, all male (mean baseline 1098 ±1107.2U/L, mean peak 886.3 ±576.6 U/L). In 4/5 subjects, both baseline and peak post-seizure were elevated.



Figure 6.5 Individual profile plots detailing changes in expression of peripheral blood high mobility group box-1 (HMGB1) following inpatient seizure in patients with drug resistant epilepsy.

Table 6.8 Baseline and peak post-seizure high mobility group box-1 and creatinekinaseinpatientsexperiencinganinpatientseizureonvideo-electroencephalography

	Baseline	Peak	Hours	Index	Baseline	Post-	Elevated
	HMGB1	HMGB1	following	seizure	CK U/L	seizure	(Y/N)
			seizure	Туре		CK U/L	
1	7.52	6.43	6	CPS	ns	65	Ν
2	4.92	7.03	3	CPS	70	55	N
3	7.89	6.76	12	CPS	65	55	Ν
4	11.25	24.37	12	CPS	100	75	Ν
5	9.13	14.33	6	SGTCS	1250	785	Y
6	12.82	18.62	24	SGTCS	80	60	N
7	17.75	10.24	3	CPS	105	75	Ν
8	10.51	12.44	24	CPS	105	80	Ν
9	9.02	12.69	3	CPS	120	90	Ν
10	9.30	24.92	3	SPS	320	270	Ν
11	13.21	12.35	12	PGS	45	95	Ν
12	8.78	12.77	24	CPS	175	85	Ν
13	7.43	9.76	24	CPS	30	30	Ν
14	9.73	18.74	3	CPS	80	85	Ν
15	4.43	4.49	12	CPS	70	60	Ν
16	5.20	6.38	24	SGTCS	305	340	Y
17	9.56	8.87	24	CPS	50	50	Ν
18	7.24	8.95	12	SPS	55	40	Ν
19	9.71	9.15	6	SGTCS	305	240	Ν
20	8.63	9.08	24	CPS	75	65	Ν
21	7.53	8.92	6	CPS	ns	25	Ν
22	8.47	9.16	24	CPS	45	55	Ν
23	10.09	10.17	6	CPS	35	35	Ν
24	8.44	9.44	6	CPS	55	35	Ν
25	8.27	10.33	12	SPS	1475	1475	Y
26	8.05	9.18	6	CPS	50	75	Ν
27	8.94	9.50	12	PGS	40	55	Ν
28	9.46	9.95	24	CPS	ns	35	Ν
29	9.47	10.73	6	SPS	45	60	Ν
30	20.97	9.79	12	SGTCS	95	175	Ν
31	18.64	10.67	3	CPS	85	55	Ν
32	12.72	10.49	24	CPS	50	40	N
33	9.16	9.57	12	SPS	65	60	Ν
34	10.89	9.25	12	CPS	90	95	Ν
35	9.64	10.58	24	CPS	60	45	Ν
36	10.54	9.87	12	PGS	20	35	N
37	9.78	11.66	3	CPS	45	55	N
38	10.34	8.95	3	CPS	30	25	N
39	10.31	21.50	24	CPS	45	40	N

40	10.35	11.11	3	CPS	110	65	N	
41	11.12	10.09	12	CPS	40	45	Ν	
42	8.33	9.34	24	SGTCS	ns	50	Ν	
43	12.24	9.85	12	CPS	90	335	Y	
44	10.40	9.61	3	PGS	290	175	Ν	
45	8.25	11.12	3	CPS	20	60	Ν	
46	12.24	9.85	3	CPS	90	55	Ν	
47	5.353	6.581	1	SGTCS	155	55	Ν	
48	6.874	12.163	3	SGTCS	155	120	Ν	
49	8.593	25.591	2	CPS	40	30	Ν	
50	3.179	3.564	6	CPS	50	40	Ν	
51	2.453	2.068	2	SGTCS	2835	1495	Y	
52	1.684	2.709	6	CPS	135	150	Ν	
53	1.556	3.479	4	CPS	170	170	Ν	
54	3.821	4.291	8	SGTCS	20	20	Ν	

HMGB1: High-mobility group Box 1; CK: Creatine kinase; Y: Yes; N: No; CPS: Complex partial seizure; SGTCS: Secondarily generalized tonic clonic seizure; PGS: primarily generalized seizure; SPS: Simple partial seizure

Variable	Ν	p-value
Age	59	0.012
Gender	59	0.349
Square Root Epilepsy	58	0.321
Duration		
Log Seizure Frequency	59	0.849
Log Index Seizure Duration	50	0.871
Epilepsy Type	59	0.314
MRI Abnormal	58	0.269
EEG Abnormal	58	0.665
Number of AEDs	58	0.243

#### Table 6.9 Testing association with log-peak-post seizure high mobility group box-1.

All continuous variables tested for association using linear regression, binary variables tested for association using t-test and categorical variables tested for association using ANOVA

# 6.4 Discussion

This study has identified for the first time that novel, circulating isoforms of HMGB1 may serve as mechanistic biomarkers for established drug-resistant epilepsy in humans. The appearance of the cytokine-activating disulphide form late in the disease

process may represent maturation of HMGB1 in the peripheral system and persistence of a functionally relevant pathological isoform.

A complex interaction of excitatory and inhibitory potentials governs the degree of neuronal excitability. Pro-inflammatory cytokines affect this balance via two main processes: alteration of voltage and receptor-gated ion channels (Viviani et al., 2007) and/or changes in glutamate and  $\gamma$ -aminobutyric acid receptor expression (Viviani et al., 2007; Vezzani et al., 2011d). Increasingly, evidence from both experimental models (Pavlovsky et al., 2005; Seiffert et al., 2004; Tomkins et al., 2007; van Vliet et al., 2007e) and human epilepsy (Marchi et al., 2007; Tomkins et al., 2008; van Vliet et al., 2007a) support the notion that disruption of the BBB sets in motion a cascade of downstream events leading to the development of an epileptic focus. Brain extravasation of serum-derived albumin following SE occurs in human TLE and the extent of BBB disruption correlate with seizures in rats (van Vliet et al., 2007a). Subsequent transformation and proliferation of neighbouring astrocytes during early epileptogenesis is a pathological hallmark in many patients with TLE (reviewed in (Binder and Steinhauser, 2006; Wetherington et al., 2008; Heinemann et al., 2000)). Cytokine release from perivascular glia has been implicated in reduced Kir234.1 channels (Binder et al., 2006; Djukic et al., 2007; Kivi et al., 2000; Schroder et al., 2000), crucial for regulation of the brain's extracellular potassium. Impaired buffering contributes to seizures by enhanced synaptic plasticity with increased neurotransmitter release and NMDA receptor activation. A disrupted BBB permits communication between the peripheral and central immune system. Up-regulation of HMGB1 and IL-1 $\beta$ /IL-1R1 is evident in neurons and glia from surgical specimens of drug-resistant epilepsy (Tomkins et al., 2007; Ravizza et al., 2008a; Boer et al., 2008; Cedazo-Minguez and Winblad, 2010; Curia et al., 2008; Ravizza et al., 2006a). Through shared pathways, both HMGB1 and IL-1 $\beta$  drive activation of neuronal IL-1R1 which induces Src kinase-mediated tyrosine phosphorylation of the NR2B subunit of the NMDA receptor. The consequent neuronal calcium influx results in seizures (Balosso et al., 2008; Viviani et al., 2003).

Sterile inflammation, driven by damage-associated molecular patterns (DAMPS), represents a physiological mechanism to protect and repair brain tissue after injury. In

drug-resistant epilepsies arising as a consequence of brain insult, DAMPs including disulphide HMGB1, may induce a persistent pathological inflammation which exceeds this natural homeostasis, turning a once protective repair into a pathologically hyperexcitable neuronal focus. Indeed, persistent pathological isoforms may represent inadequate anti-inflammatory mechanisms in some individuals, which may have a genetic origin. A disrupted BBB permits communication between the peripheral and central immune system. It is increasingly evident that neuroinflammatory processes are key to establishing a chronic epileptic focus following epileptogenesis (Vezzani et al., 2011a). In this study of patients with long-standing drug-resistant epilepsy, elevated serum HMGB1 may have two possible origins. Either spill-over from the CNS of brain-derived HMGB1 and/or a peripheral immune response to an epileptogenic insult wherein HMGB1 crosses the disrupted BBB for the purpose of repair.

The diagnostic performance of a test or the accuracy of a test to discriminate diseased cases from normal cases was evaluated using ROC curve analysis. For any given population with a disease, it is rare to observe a perfect separation between diseased and healthy groups. The ROC curve plots the true positive rate (sensitivity) as a function of the false positive rate (100-specificity), reviewed in detail in chapter 1. A test with a perfect discrimination between diseased and healthy will have a curve that passes the upper left corner of the graph, which is 100% sensitive and specific. Thus the closer the curve to the top left, the higher the overall accuracy. ROC curve analysis in this study supports the notion that HMGB1 could provide a measure of separation between drug responsiveness and drug resistance in patients with established chronic drug resistance. However, the ability of HMGB1 to predict resistance at first presentation remains to be elucidated.

In this study, abnormal MRI, defined as the presence of hippocampal sclerosis, cortical dysplasia, vascular lesions, gliosis and/or abnormal increased flair signal at the focal site, was associated with elevated baseline HMGB1. The MRI abnormalities described are aetiologies that are often considered for surgical treatment and have been shown to carry an almost six-fold risk of persistent seizures compared with cryptogenic epilepsy, in which the cause is unknown (Liimatainen et al., 2008). An observational study of over 2200 patients with epilepsy found that hippocampal sclerosis (HS),

cerebral dysgenesis, and dual pathology (HS and another lesion) were associated with a low rate of seizure-freedom (11%, 24%, and 3%, respectively) (Semah et al., 1998). The presence of a neurological insult was found to be a significant risk factor for time to treatment failure in the SANAD study, a randomised controlled trial in which standard antiepileptic drugs were compared with new treatments (Bonnett et al., 2012). This suggests that abnormal imaging is an indicator of increased risk of drug resistance and a need for earlier consideration of surgical treatment. Epilepsy surgery ranges from focal resection of the epileptogenic cortex (antero-mesial temporal lobe and other focal cortical resections) to interventions that remove or isolate the cortex of a grossly diseased hemisphere. It is majorly underutilized, in the United States of America, under 1% of patients with drug-resistant epilepsy are referred to epilepsy centers (Engel, 2013). The largest and longest prospective study of epilepsy surgery, covering outcome data annually until 19 years post-surgery, validated the long-term effectiveness of epilepsy surgery showing continuous seizure free epilepsy in over 50% of patients. In this study, the average delay from diagnosis to surgery was 20 years (de Tisi et al., 2011), highlighting the need to consider and refer for surgery at an earlier stage in appropriate candidates. A mechanistic biomarker, able to stratify early those at the greatest risk of drug-resistance, could significantly aid the physicians decision to refer early. At early post-surgical follow-up, freedom from seizures has been reported in 60-70% of patients (J Engel Jr, 1993; Wiebe et al., 2001; Tellez-Zenteno et al., 2005); however this should perhaps be viewed with a note of caution, as some studies report seizure freedom rates declining to 40-50% at 10 years (McIntosh et al., 2004; Dunlea et al., 2010; Salanova et al., 1999; Dupont et al., 2006; Foldvary et al., 2000). Therefore, the need for better pharmacotherapy is not diminished by the availability or suitability of epilepsy surgery.

HMGB1 is ubiquitous in cells and can be released as after muscle injury. HMGB1 expression was elevated in skeletal muscle after hind-limb ischaemia in a murine model of muscle injury (Lin et al., 2005). A recent review undertaken to assess the sensitivity and specificity of CK to differentiate between epileptic seizure and psychogenic non-epileptic seizure (PNES) determined that a marked rise in CK, above the 95.7<sup>th</sup> percentile, has a high specificity, but low sensitivity, for the diagnosis of

epileptic seizure. However, limited data is available to determine whether CK can differentiate between convulsive and non-convulsive seizure (Brigo et al., 2015). In the present study, elevated CK was identified in 5 patients experiencing a seizure, 3 of which were secondarily generalized, one was a complex partial seizure and the other a simple partial seizure. In only 2 cases CK was elevated >4 times above the normal range. Serum CK levels have been shown to rise markedly following generalized seizures, usually with a delay of at least 3 hours and with a peak concentration occurring after more than 36-40 hours (Wyllie et al., 1985; Chesson and Kasarskis, 1980; Chesson et al., 1983). The mean peak-HMGB1 time following seizure was  $11.4 \pm$ 8 hours, and therefore potentially CK levels could continue to rise as a consequence of seizure activity and later time point analysis would be required to fully exclude this possibility. However, the majority of patients in this study had frequent, recent seizures and therefore one would expect high baseline levels of CK in all the subjects if that were to explain the persistence of the pathological isoform. Indeed, 23/65 subjects had a seizure (any type) within 24 hours of the admission. Furthermore, for those experiencing regular generalized tonic clonic seizures (14/59), mean monthly seizure frequency in this group was 12.2 ± 22.7, therefore; it is unlikely that persistence of the inflammatory isoform in the drug resistant group can be explained by recurrent muscle injury related to frequent seizure activity, as one would expect much higher baseline CK. Persistence of the inflammatory disulphide-HMGB1 in patients with drug-resistant epilepsy and frequent seizures cannot therefore be attributed solely to muscle damage as a consequence of seizures.

Initially in this study, recruitment was restricted to a single site in the UK. Recruitment to the study was slower than expected, resulting from the vast majority of requests for inpatient video EEG telemetry originating from diagnostic uncertainty, particularly where Non-epileptic attack disorder (NEAD) was suspected. Opening a second site for recruitment in the UK was not possible; other UK-based video-EEG sites were approached (London) but due to the relative labour-intensive nature of the study, particularly for collection of post-seizure samples out of hours, a second site could not be found. In order to obtain sufficient samples to examine the relationship between seizures and HMGB1 in epilepsy, we collaborated with the epilepsy research group in Tampere, Finland, who had previously undertaken a serial-sampling inpatient EEG study examining other peripheral inflammatory biomarkers. The study design shared several similarities, in particular the classification of epilepsy and seizure, inclusion of drug-resistant pre-surgery patients, inclusion of focal and non-focal epilepsies and exclusion of patients with inter-current illness or co-morbidities. Sufficient similarities existed for the patient groups to be considered comparable. However, the post-seizure sampling time frame for the two studies differed. Initial design of the UK study involved intense sampling immediately following seizure and at 30 minute intervals. As more information about HMGB1 because available over time, it was realized that as a late mediator of inflammation, it would be prudent to collect samples at later time points. An amendment to the study with ethical approval was made and as a result, some of the earlier UK patients had missing data for samples at later time points (>6 hours). The study in Tampere identified significantly higher baseline highly-sensitive CRP in patients with drug-resistant epilepsy temporal lobe epilepsy compared to healthy controls (Alapirtti et al., 2012). The authors also found that a significant increase in CRP from baseline was prompted by secondarily generalized tonic-clonic seizures. The present study found no association between HMGB1 and the elevated CRP found in the patients with TLE. Furthermore, in the majority of patients, HMGB1 did not rise significantly further following generalized tonic–clonic seizure, in keeping with the absence of elevations in CK, further suggesting that the high baseline HMGB1 is not related to the muscular activity component seizure. HMGB1 is known to be a late mediator of systemic inflammation, particularly in clinical sepsis, where serum HMGB1 levels increased significantly (Wang et al., 1999).

Inflammatory processes in the CNS are increasingly accepted as key contributors in the aetiopathogenesis of epilepsy. As an inflammatory mediator implicated in the mechanisms of seizure generation, HMGB1 shows promise as a novel translational biomarker able to stratify drug-responsiveness from drug-resistance in patients with established epilepsy. Future studies are now required to examine HMGB1 in those with first isolated seizures of varying aetiology and newly diagnosed epilepsy to determine whether it has any prognostic value. Furthermore, a subset of drug-resistant patients was identified expressing the disulphide form of HMGB1. No

association with any clinical variable was found, suggesting that this particular biomarker may be a novel means to stratify those most amenable to immunomodulatory therapy. Currently available AEDs suppress the clinical expression of seizure but have no impact on disease progression. Failure to control seizures occurs in up to 40% of patients, further complicated in many by intolerable adverse effects that limit the attainment of therapeutic dosage. Next generation AEDs need to possess antiepileptogenic properties. Parallel development of mechanistic biomarkers able to predict progression of disease and treatment response would be invaluable for drug discovery in this area. Monoclonal antibodies targeting HMGB1 are already showing considerable success in other preclinical models of disease (Kokkola et al., 2003; Schierbeck et al., 2011; Yang et al., 2006; Chorny and Delgado, 2008). It may be the case that some isoforms of HMGB1 are protective and therefore development of isoform-specific antibodies is the next logical goal.

In the presence of normal brain MRI and EEG, currently the physician's ability to predict both recurrences after first seizure and treatment response is severely limited. Arguably, no patient group would benefit more substantially from identification of circulating biomarkers of disease progression and treatment response than those with epilepsy. Further studies examining the prognostic value of HMGB1 isoforms in first presentation of seizure are certainly warranted. 

# **Final Discussion**

The overall aim of this thesis was to examine the potential for blood HMGB1 to act as a mechanistic biomarker in epilepsy, particularly for the early identification of drug resistance. Overexpression of HMGB1 and its receptor TLR4 has been identified in brain tissue in both experimental models and humans with drug resistant epilepsy (lori et al., 2013; Maroso et al., 2010; Maroso et al., 2011b; Zurolo et al., 2011). In rodents, exogenous application of HMGB1 prior to chemoconvulsant challenge exacerbates seizures. Furthermore, selective inhibition of HMGB1 or TLR4 delays seizure onset and decreases seizure number and duration (Maroso et al., 2010). HMGB1 undergoes extensive post-translational modifications which dictate its inflammatory functions. In mice, the disulphide-containing cytokine isoform of HMGB1 has been shown to increase neuronal cell death *in vitro* and exacerbate kainate-induced seizures *in vivo* (Balosso et al., 2014). The mechanism involved is tyrosine phosphorylation of the NR2B subunit of the NMDA receptor, which ultimately enhances Ca<sup>2+</sup> influx in neuronal cell bodies (Balosso et al., 2014). Taken together, these studies provide proof-of concept that HMGB1 plays an important role in the development of seizures and epilepsy.

The results of the thesis reveal that peripheral blood HMGB1 exhibits low baseline variability in health, is detectable following experimental seizures in rodents in a model independent fashion and is over-expressed in peripheral blood in patients experiencing regular seizure activity. , Presence of the acetylated isoform stratifies resistance (regular seizures) from responsiveness (absence of seizure activity), and a subset of resistant patients express the pathological disulphide form which may indicate aberrant inflammation in some.

Diagnosis and treatment of epilepsy suffers from a lack of reliable biomarkers. Currently, there is no way to predict whether epilepsy will occur following potential epileptogenic insults to the brain. What is more, the diagnosis of epilepsy is based on clinical criteria that appear once seizures develop, but the pathophysiological process may begin years before. There is no way to diagnose epilepsy prior to the occurrence of seizures and in most cases; treatment is not commenced following first seizure until further seizures occur. The pharmacological management of epilepsy involves a degree of trial and error of the AED therapeutic arsenal. And even after treatment commences, there is no definitive means to predict the development of drugresistance, which is seen in about 30% of patients. In addition, development of disease-modifying anti-epileptogenic drugs is hampered by a lack of understanding of the mechanisms underlying the development of an epileptic focus following brain insult. Early in drug development, biomarkers to demonstrate target engagement and proof of mechanism are of high value. What is more, such biomarkers are also of importance in phase III clinical trials, to label a drug as having disease-modifying effects. Therefore there are several areas wherein biomarkers would enhance epilepsy management including:

- 1.) Prediction of epilepsy following brain insult
- Prediction of epilepsy following first seizure; to differentiate between a reactive, unprovoked isolated seizure and definitive epilepsy.
- 3.) Prediction of response to therapy (resistant versus responsive) to facilitate early alternative therapy, such as surgery, avoiding prolonged drug trials with associated cost and morbidity.
- 4.) Facilitate early pre-surgical evaluation.
- 5.) Improve drug discovery by streamlining screening of potential new AEDs.
- 6.) Reduce clinical trial costs by enriching patient populations.
- Marker of epileptogenicity to determine definitively that an epilepsy condition has been a) prevented or b) cured.

The findings outlined in this thesis are therefore timely, and contribute to the growing body of literature.

Focal epilepsy arises as a result of some form of insult to the brain, such as hypoxic brain injury or prolonged febrile convulsion in childhood (Shinnar et al., 2008; Volpe, 2008; Dinner, 1993). As a consequence, a normal physiological acute inflammatory reaction occurs with the subsequent release of inflammatory mediators including IL-1 $\beta$  and HMGB1. Both have been shown to promote neuronal damage and exacerbate seizures in various pre-clinical seizure and epilepsy models (Viviani et al., 2003) and both have been shown to be up-regulated in human brain tissue at epilepsy surgery (Zurolo et al., 2011). In addition, both can trigger the release of the other, potentially setting up an auto-feedback loop for prolonged release (Steer et al., 2006; Keyel,

2014). Through mechanisms already discussed (1.7.3), HMGB1 can lower the seizure threshold from whence a clinical seizure arises. It may be the case, in some individuals, that this initial neuroprotective inflammatory response to insult fails to resolve. Some individuals may continue to produce pathological mediators of inflammation, including the disulphide form of HMGB1, that aggravate the initial insult leading to a permanent state of localised hyperexcitability resulting from excessive intracellular Ca<sup>2+</sup> influx and glutamate release. In this way, pathological isoforms of HMGB1 may be integral to the initial period of epileptogenesis following brain insult and indeed to the maintenance of the epileptic state. Both centrally, through localised release in brain, and peripherally, by activation of the peripheral immune response.

In the early post-brain insult or seizure phase, passive release of nonacetylated/reduced HMGB1 in the periphery may originate from muscular insult resulting from ongoing seizure activity. This could be followed, in some individuals, by a late phase production of the inflammatory acetylated/disulphide isoforms, triggered in response to the initial muscular insult. Frequency of seizure activity at baseline is associated with a greater risk of developing drug resistance (Hitiris et al., 2007; Mohanraj and Brodie, 2006), raising the possibility that there may be a critical threshold in the biomarker level, over which point the late phase of inflammatory HMGB1 may be triggered. Indeed, in peripheral blood, in two independent seizure models (KA, chapter 4 and MES, chapter 5) non-acetylated, reduced HMGB1 showed early release consistent with necrosis and functional chemotaxis for leukocyte recruitment and repair. An enduring disruption to the BBB, as is postulated to occur in epilepsy (reviewed in detail in 1.5.1), potentially permits a bi-directional link between the peripheral and central immune system, allowing peripherally generated HMGB1 to cross into the CNS and aggravate the epileptogenic focus. However, despite examination in different disease states and biofluids in humans and different preclinical models of seizures and epilepsy (KA, MES and pilocarpine), no direct link between central levels of HMGB1 and peripheral levels was identified. No correlation between CSF and serum HMGB1 was identified in any condition examined (headache, neuroinfection or Rasmussen's encephalitis). Examination of paired serum and CSF HMGB1 samples taken from patients with epilepsy is now required, in order to determine whether there is a direct relationship between the compartments. Through an existing collaboration, this work is currently underway to collect paired samples in newly diagnosed epilepsy. Reports of elevated CSF biomarkers for the detection of subtle pathological insults following seizures have been made. The cytoplasmic enzyme neuron-specific enolase (NSE) is part of the glycolytic pathway for the conversion of glucose to pyruvate and is essential for cellular energy metabolism (Marangos et al., 1978; Zomzely-Neurath, 1982). It is regarded as a marker of acute brain injury in stroke, global ischemia, and coma. CSF-NSE levels have been shown to be elevated compared with the levels for normal control subjects in patients within 24 hours of experiencing SE. Elevated CSF-NSE also correlated with elevations in the CSF/serum albumin ratio, a measure of BBB integrity (Correale et al., 1998). Elevated CSF-NSE has also been reported in children experiencing non-febrile seizures (Ko et al., 1990) and in adults with epileptic seizures (Royds et al., 1983), but the numbers studied were small. More recently, in 22 patients with newly presenting, untreated generalised seizure, no significant difference between CSF and serum NSE compared to neurologically normal controls was demonstrated within 24 hours of the seizure (Palmio et al., 2001). This likely reflects a difference in pathology; prolonged SE may lead to hippocampal damage whereas, short, uncomplicated seizures, however frequent, may not result in neuronal damage sufficient to cause release of a biomarker that reflects acute brain injury. However, in the case of seizure disorders, repeated convulsive seizure activity may lead to the continual, low-grade production of HMGB1, either centrally or peripherally, with consequent aggravation of the initial underlying brain insult. Therefore one would expect a direct link between CSF levels and peripheral blood levels, resulting either from spill-over from the CNS across a disrupted BBB, or peripheral production crossing into the CNS. However, it may be the case that continued production of HMGB1 occurs only in some individuals, those that go on to develop poor control, and therefore longitudinal follow-up of these patients will be required.

Two weeks following the induction of SE with KA, acetylated and disulphide HMGB1 was significantly elevated, likely resulting from immune activation. The cause of this delayed immune activation is not currently known. It is possible that the appearance of

spontaneous convulsive seizures in the mice led to low-grade production of nonacetylated/reduced HMGB1 from convulsive muscle injury. As a result, delayed maturation of the inflammatory isoforms occurs, which may then have a pathological role. Failure to detect the appearance of the non-acetylated/reduced forms may reflect the time points selected (7 and 14 days) and more frequent sampling would be required to identify this. The pilocarpine model of epilepsy (chapter 5) demonstrated significantly higher total serum HMGB1 in epileptic animals; however no significant elevation occurred in brain (as assessed by western blotting.) At present, this argues against the theory that central HMGB1 passes over into the peripheral blood however extracellular release of the protein has yet to be determined by LCMS/MS, and this analysis is currently underway. In contrast, in the KA model, significantly elevated total intracellular hippocampal HMGB1 persisted until 14 days in concert with elevated peripheral expression. However, extracellular release, determined by LCMS/MS, did not show a corresponding elevation in brain. The discrepancy between the models may in part reflect the timing of sampling; both models examined the relative early time course of the disease process when major epileptogenic changes are underway. Subtle changes in expression may have been missed due to the wide time course over which sampling occurred (3, 7 and 14 days in KA and 28 days in pilocarpine.) Further limitations of the pilocarpine model include its restriction to the detection of purely convulsive seizures. Movement plates within the home cages detected convulsive seizure activity and the video correlate was used to confirm the presence of a generalised seizure. Periods of non-convulsive seizure activity and focal, limbic seizures are not detected in this model. Therefore, further work is underway through an existing collaboration utilising a chronic epilepsy model with continuous video-EEG surveillance to capture all periods of abnormal brain activity which can then be related to biomarker expression. In addition, early epileptogenic periods will be analysed alongside the true chronic epileptic phase occurring 5 months following the initial brain insult, in an attempt to address the unanswered questions highlighted by the present models. Particularly, whether there is a direct link between hippocampal expression of HMGB1 isoforms at the site of injury and the subsequent levels released into, or produced by, peripheral blood.

Analogous to the findings in the KA model, patients with ongoing seizures expressed only the acetylated form of HMGB1, while a sub-cohort of those expressed both reduced and disulphide isoforms (chapter 6). Current clinical evidence to suggest that counteracting inflammation is therapeutically beneficial in adult epilepsy in humans is limited (Walker et al., 2013). However, with growing evidence to support its potential contribution to the generation of seizures, and possibly to epileptogenesis itself, antiinflammatory agents can be considered as candidates in the ongoing search for novel AEDs. The compounds arguably showing greatest promise, and furthest down the development pipeline, are inhibitors of interleukin converting enzyme (ICE)/caspase-1, the protease that catalyses the conversion of the inactive precursor pro-IL-1 $\beta$  to active IL-1 $\beta$  (Kuida et al., 1995). Inhibition of ICE/caspase-1 reduces the release of IL-1 $\beta$  in organotypic hippocampal slices following exposure to pro-inflammatory stimuli (Ravizza et al., 2006e), decreases acute seizure activity following intrahippocampal kainate in rats (Ravizza et al., 2006e), and restricts the generalization of seizures in a rapid kindling model (Ravizza et al., 2008a). These effects are closely correlated with a reduction in the expression of IL-1 $\beta$  in hippocampal astrocytes. They are unsurprisingly absent in mice in which the corresponding gene has been knocked out and that consequently display an inherent resistance to experimental seizures (Ravizza et al., 2006e). In addition, IL-1 $\beta$  signalling can be blocked via its natural antagonist, interleukin-1 receptor antagonist (IL-1Ra). Human recombinant IL-1Ra (Anakinra) is licensed for the treatment of moderate to severe rheumatoid arthritis (Food and Drug Administration, 2001a). Anakinra competitively inhibits the binding of IL-1 $\alpha$  and IL-1 $\beta$ to the IL-1 receptor and thus inhibits the effects of this pro-inflammatory cytokine (Urien et al., 2013). Such targeted anti-IL-1β interventions have led to a considerable reduction of seizures in various models in seizure and epilepsy (Akin et al., 2011; Vezzani et al., 2002; Vezzani et al., 2000; Vezzani et al., 1999; Ravizza et al., 2008h; Ravizza et al., 2006e; Maroso et al., 2011b; Maroso et al., 2010; Auvin et al., 2010a). In addition, blockade of IL-1 and TLR4 has been shown to be neuroprotective (Allan et al., 2005; Ross et al., 2007; Vezzani et al., 2013). Systemic administration of VX-765, a prototypic ICE/caspase-1 inhibitor, increased the time to seizure onset and decreased cumulative duration of electrographic seizures induced by acute intrahippocampal kainate; whilst in the chronic model, VX-765 decreased the time spent in spontaneous

epileptic activity by up to 75%. This anticonvulsant action was again correlated with a reduction in the expression of IL-1 $\beta$  in hippocampal astrocytes and microglia (Maroso et al., 2011a). More recently, in two independent rat models of epilepsy, pharmacological blockade of IL-1β-mediated signalling, using IL-Ra and VX-765, afforded significant neuroprotection in the form of decreased IL-1 $\beta$  expression in astrocytes and cell loss in rat forebrain (Noe et al., 2013). Through collaboration (Vezzani, unpublished data) we investigated the effect of pharmacological blockade of IL-1β-mediated signalling on HMGB1 biomarker expression in an electrically-induced SE model of epilepsy (details of model in appendix). Five months following electricallyinduced SE, epileptic seizures were associated with persistence of the acetyl and disulphide inflammatory forms of HMGB1. In the treated rats only, complete prevention of the disulphide, cytokine-stimulating form of HMGB1 in peripheral blood was seen from 15 days following the initial SE and persisting at 5 months (unpublished data in appendix). These findings confirmed the previously reported efficacy of VX-765 in preclinical models, further supports its proposed mechanism of action, and suggest that this class of compounds merits further evaluation as putative AEDs.

VX-765 (developed by Vertex Pharmaceuticals Incorporated) was originally developed for the treatment of inflammatory and autoimmune conditions (Randle et al., 2001). It is a pro-drug with good oral bioavailability, whose active metabolite, VRT-043198, is known to cross the BBB following systemic administration, making it an attractive candidate for the treatment of CNS disorders with a proposed inflammatory component. A phase 2a study was completed in drug-resistant partial epilepsy (Vertex, 2011), the results of which suggest that it is safe and well tolerated when administered over a 6-week period. In terms of preliminary efficacy data, the mean percent reduction in seizure rates were 15.6% in the VX-765 group compared to 7.0% in the placebo group with a  $\geq$ 50% reduction in seizures in 18.8% of subjects in the VX-765 group versus 8.3% in the placebo group. However, the response rates did not meet statistical significance (Bialer et al., 2013). A 13-week dose-ranging phase 2b trial was prematurely curtailed following a business-related decision from Vertex in 2012 (Bialer et al., 2013). Response rates for the 20 subjects already enrolled will eventually be available but the study will be substantially underpowered. A future opportunity may be to undertake further studies by stratifying patients based on a biomarker such as HMGB1.

Agents targeting early pro-inflammatory cytokines have proven ineffective in treating sepsis, even in large-scale clinical trials (Abraham et al., 1998; Fisher et al., 1994). A major difficulty in developing therapeutics that target cytokines such as TNF $\alpha$  and IL-1 $\beta$ is that they are released early in the development of a systemic inflammatory response (Tracey et al., 1986), leaving a very narrow therapeutic window for administration of an effective antagonist. In the case of focal epilepsies, where inflammation-driven epileptogenesis occurs over a latent period following brain insult, a broader therapeutic window is required. Therapeutic strategies targeting late-acting, clinically accessible mediators, such as HMGB1, would be preferable. Experimental sepsis induced by caecal perforation reveals this broader therapeutic window in which HMGB1 functions in action. Anti-HMGB1 antibodies and ethyl pyruvate, a simple aliphatic ester of pyruvic acid with proven anti-inflammatory action (Shin et al., 2014a), rescue mice from otherwise lethal sepsis even when administered 24 hours following onset (Ulloa et al., 2002; Yang et al., 2004). Anti-HMGB1 antibodies have high molecular weights which may prevent their access to the intact brain. However, disruption of the BBB, now seen as pathognomonic in seizures, potentially provides a unique opportunity for delivery of anti-epileptogenic antibodies into the brain. A fully humanized anti-HMGB1 antibody would be required prior to moving into the clinic. In addition, ethyl pyruvate has been shown to inhibit HMGB1 phosphorylation and release in activated microglia following experimental stroke (Shin et al., 2014b).

Several small molecules have been investigated in recent years for their ability to inhibit HMGB1 activity. *Glycyrrhizin* is a natural triterpene found in roots and rhizomes of liquorice (Glycyrrhiza glabra) (Mollica et al., 2007). Glycyrrhizin inhibits the chemoattractant and mitogenic activities of HMGB1 and has a weak inhibitory effect on its intranuclear DNA-binding function (Mollica et al., 2007). It has been investigated in pre-clinical models for its anti-HMGB1 properties in many conditions including liver injury (Ogiku et al., 2011; Gwak et al., 2012), sepsis (Wang et al., 2013), colitis (Vitali et al., 2013) and intracerebral haemorrhage (Ohnishi et al., 2011). In the post-ischaemic mouse brain, administration of intravenous Glycyrrhizin after occlusion of the middle cerebral artery was neuroprotective and associated with almost complete cessation of HMGB1 secretion compared to untreated control (Kim et al., 2006). A similar neuroprotective effect was seen in the spinal cord injury model in rats, with reduced release of inflammatory cytokines along with HMGB1 inhibition (Gong et al., 2012). Given its preclinical success in a wide range of conditions, further exploration of glycyrrhizin and structural analogues is warranted in the search for effective HMGB1 inhibitors.

Growing evidence suggests that statins, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, exhibit a neuroprotective action in an array of neurological diseases including stroke, Alzheimer's disease, Parkinson's disease and epilepsy (van der Most et al., 2009; Stepien et al., 2005; Reiss and Wirkowski, 2009). In addition to their lipid lowering activity, statins exert pleiotropic effects and their antiinflammatory properties are well established. Both atorvastatin and simvastatin afford neuroprotection from ischemic brain injury in rodent models of stroke, including attenuation of the vascular inflammation and atherosclerotic lesion area, with decreased expression of HMGB1, RAGE, TLR4 and NF-KB (Wang et al., 2010; Liu et al., 2013). Atorvastatin and simvastatin have also been shown to improve cognitive performance in a rodent model of spatial memory (Vandresen-Filho et al., 2015), and improved biochemical and behavioural alterations in an experimental model of Parkinson's disease (Kumar et al., 2012). In cell culture, in a dose-dependent manner, atorvastatin inhibits HMGB1-induced vascular endothelial activation by reducing intercellular adhesion molecule-1 (ICAM-1) and E-selectin with resultant reduced leukocyte-endothelial adhesion (Yang et al., 2010). Elevated expression of ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), P-selectin and E-selectin has also been identified following pilocarpine-induced status epilepticus (Fabene et al., 2008), which has been implicated in BBB breakdown, a pathological step in the development of an epileptic focus. Disruption of the leukocyte-endothelial cell interaction results in a marked reduction in spontaneous seizures in this epilepsy model (Fabene et al., 2008). It is biologically plausible that the pleiotropic anti-inflammatory effects of statins, targeting leukocyte-endothelial interactions, could exhibit anti-epileptogenic effects in terms of preventing BBB leakage, an avenue that certainly warrants further exploration

given the preclinical evidence in various models of neurological disease. However, to date, the uptake, distribution and metabolism of statins in brain is not well understood. Furthermore, the dose-response relationship of statins if used in such an indication will need careful study. Conflicting data exists, a small study of CSF levels of statins following 5 days of treatment found that pravastatin did not pass the bloodbrain barrier (Botti et al., 1991). In addition, an in situ rat brain perfusion study using radiolabelled compound also concluded that pravastatin did not cross the BBB (Saheki et al., 1994). However others have found that simvastatin, lovastatin and pravastatin are all detectable in the brain of mice following 21 days of statin therapy (Johnson-Anuna et al., 2005; Thelen et al., 2006). The conclusion of the studies is that lipophilic statins cross the BBB, but are rapidly eliminated. Statins may conceivably afford a degree of anti-inflammatory and thereby neuroprotective action following brain insult which may prove anti-epileptogenic. However, further studies addressing the CNS pharmacokinetics and pharmacodynamics, and dose-response relationships, of statins in brain are first required in order to understand the mechanisms of statin-induced neuroprotection.

There are a number of outstanding questions arising from this research that require further exploration. Firstly, the true reference interval for HMGB1 in health remains unknown at present. Throughout the world, there are many ongoing studies for defining reference intervals. According to the IFCC guidelines, reference intervals for analytes are defined as the central 95% of values from a reference population (minimum 120 individuals). Total HMGB1 and the relative isoform expression will be determined in 200 healthy individuals through the healthy control arm (n=200) of the ongoing BIOPAR study (Antoine et al, unpublished data), a longitudinal observational study examining biomarkers in paracetamol overdose. Secondly, this thesis identified, for the first time, overexpression of HMGB1 in CSF and blood in Rasmussen's encephalitis. Further analysis is required to examine the isoforms present in order to determine both the mechanism of release (necrotic versus inflammatory) and the functional role (cytokine, chemotaxis or immunologically inert) of the different isoforms of HMGB1 in this condition. This may contribute to the understanding of the pathological course of RE, particularly the proposed inflammatory mechanism. In
addition, HMGB1 isoform analysis in CSF and blood in neuroinfection is required. Potentially, both central (e.g. microglia) and peripheral (e.g. white blood cells then invading the CNS) production of HMGB1 is possible in neuroinfection. Identification of the isoforms present may prove a useful tool for stratification in neuroinfection and for the identification of therapeutic targets. Thirdly, further analysis of the isoforms present is needed in the epileptic mice with spontaneous seizures to identify whether the expression reflects the findings in the human study. Specifically, whether the mice express the acetylated form (seen in drug-resistant humans) or the non-acetylated form (seen in those who are seizure free.) This would clarify whether the model reflects the drug-responsive or drug-resistant phenotype of the human disease and would serve as a platform for further investigation into the pathological differences.

Activation of innate immunity and inflammatory pathways as a consequence of epilepsy is, on the basis of available evidence, almost beyond doubt. This is an important finding with therapeutic potential for the control of pre-existing seizure disorders. To date, very few studies have examined the use of immunomodulatory agents in focal epilepsy syndromes (Walker et al., 2013). The animal model data presented in this thesis supports the existing literature that inflammatory pathways involving HMGB1/TLR4 and the IL-1 $\beta$ /inflammasome axis are causally involved in epileptogenesis. However, whether there is a direct link between brain and serum in epilepsy requires further evaluation. The results of this thesis suggest, in different animal models of seizure and in different disease states in man, that there is no direct correlation. Further analysis is ongoing to clarify some of the gaps in the knowledge highlighted by these studies. Examination of brain tissue taken from patients with drug-resistant epilepsy (Vezzani et al., 2011a) is limited by the absence of comparative control tissue, specifically that people with well-controlled epilepsy do not undergo resective epilepsy surgery. Therefore it is not possible to know whether the overexpression of inflammatory mediators identified in drug-resistant brain tissue occurs as a cause/consequence of the epileptic state or drug-resistance, or perhaps both. A potential means to address whether biochemical markers of inflammation reflect central inflammation, and whether or not this is relevant to both wellcontrolled and poorly-controlled epilepsy, would include advanced neuroimaging of

inflammation. Future studies, examining the prognostic capability of HMGB1 isoforms as a biomarker of drug-resistance, should include imaging modalities able to identify neuroinflammation. This may include positron emission tomography or magnetic resonance spectroscopy, able to examine cell turnover and microglial activation through assessment of choline and myo-inositol. Immunohistochemical evidence of HMGB1 activation (nucleus to cytoplasmic shift) has recently shown correlation with both very high-field and specific MRI and also a deoxyhaemoglobin-sensitive T2\* sequence in a rat model of febrile status epilepticus (Choy et al., 2014).

Given the complex, multifactorial nature of epilepsy and epileptogenesis, it seems unlikely that a single biomarker will be able to predict both the development of epilepsy following first seizure and the risk of drug-resistance, in addition to assessing epileptogenicity. It is more likely that a panel of biomarkers rather than one single marker will meet these criteria in the future. That being said, HMGB1, through its involvement with inflammasome assembly and its intrinsic link to IL-1 $\beta$  activation and release, is uniquely placed to serve as an anchor for future exploratory studies of inflammatory biomarkers in epilepsy. In addition to patient stratification, HMGB1 shows promise as a novel therapeutic target for the prevention of epileptogenesis following brain insult, with both anti-seizure and neuroprotective effects (Balosso et al., 2014). However, the long-term safety of an immunomodulatory intervention that targets a fundamental pathway that exists to protect rather than do harm needs to be rigorously established. This argues again for the need for mechanistic biomarkers of the disease process that can be easily measured and used as a quantifiable measure of treatment response.

The emerging data on inflammation and epilepsy represent a potentially novel avenue for drug development in epilepsy and one that is not only distinct from previous approaches but also based on sound neurobiological evidence. Biomarkers that are also putative drug targets represent the ideal to the pharmaceutical industry for companion drug-diagnostic development. However, until the mechanisms underlying epileptogenesis can be fully elucidated, including unravelling whether it occurs as a result of concurrent, overlapping or divergent mechanisms, the development of disease-modifying drugs for epilepsy remains in its infancy. However, early surgical intervention provides the best opportunity to avoid the irreversible adverse consequences of recurrent seizures. A means to definitively identify patients who have epilepsy conditions that will never respond to AEDs at the outset would still be tremendously advantageous.

As an inflammatory mediator implicated in the mechanisms of seizure generation, HMGB1 shows promise as a novel translational biomarker able to stratify drugresponsiveness from drug-resistance in patients with established epilepsy. Future studies are now required to examine HMGB1 isoforms in those with first isolated seizures of varying aetiology and those with newly diagnosed epilepsy to determine whether it has prognostic value. 

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## Appendix

	Parameter	HMGB1 (ng/ml)
Intra-assay variation	Range	178-192
	Mean	187
	SD	5.5
	CV (%)	2.9
Inter-assay variation	Range	175-192
	Mean	185
	SD	6.4
	CV (%)	3.5

Assay validation data for the serum determination of High Mobility Group Box-1 from a male CD-1 mouse dosed with paracetamol 530mg/kg for 5 hours.

Samples assayed once on 5 separate days (inter-assay variation) or 5 times on one day (intra-assay variation). Samples range, mean, standard derivation of the mean (SD) and coefficient of variation (CV) is included for analysis. HMGB1: High Mobility Group Box-1. Table adapted from (Antoine et al., 2009)

The electrical kindling rat model of epilepsy involves sustained electrical stimulation of the hippocampus or amygdala, via surgically implanted depth electrodes, to induce a period of SE characterized by recurrent focal and generalized seizures which are uninterrupted. This is followed by the development of neuropathological features consistent with human mesiotemporal sclerosis and the development of recurrent spontaneous seizures arising typically after 3-4 weeks (Loscher, 2002). Rats were divided into 3 experimental groups:

 Sham-operated rats implanted with electrodes but not electrically stimulated (Sham);

2. Rats experiencing SE and treated with vehicle;

3. Rats experiencing SE, and treated with a combination of anakinra and VX-765 (Treatment). Drugs were administered for 5 or 7 consecutive days starting 3 hours after the end of electrical stimulation.

Peripheral blood samples were obtained via the tail vein at 7, 15 and 90 days following electrical kindling.



Mass spectrometric quantification and characterization of (a.) total high mobility group box-a (HMGB1), (b.) acetylation and (c.) redox isoforms from rat sera (n=10/group) 7, 15 and 90 days following electrical kindling. Results are expressed as the mean  $\pm$ standard error of the mean, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001 by 2-way repeated measures ANOVA with Tukey correction for multiple comparisons.