Comparison of Cardioprotective Therapy with Atenolol and Diltiazem in Seizure-Induced Cardiomyopathy

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Abstract

Seizure-induced autonomic dysregulation is frequently linked with the development of cardiac abnormalities. The current study examined the therapeutic effects of pre- and post-treatment with atenolol or diltiazem in a model of seizure-induced cardiomyopathy. Male Sprague-Dawley rats (320-350g) were implanted with a telemetric transmitter to simultaneous record EEG/ECG and an intrahippocampal (ih.) drug cannula to allow delivery of kainic acid (KA; 2 nmol, 1 uL/min) directly into the hippocampus. Seizure behavioural activity was assessed using a 0-5 point modified Racine scale. Pre-treatment animal groups (n=4) were administered saline, atenolol (5 mg/kg) or diltiazem (2.5 mg/kg, bid.) subcutaneously for 3 days prior to KA-seizure induction and for a further 48 hours post-seizure. In comparison, post-treatment animals received saline, atenolol (5 mg/kg) or diltiazem (5 mg/kg bid.) subcutaneously 60 min post-KA and for the remaining 7 days.

Induction of seizures in saline dosed (saline-KA) animals resulted in increased mean heart rate (HR; 17%) over the immediate 3 hour recording period, as well as a concurrent rise in systolic blood pressure (SBP; 27%) at 1 hour post induction. Pre- and post-treatment with atenolol significantly reduced seizure-induced ECG changes, including HR and QTc interval. Animals pre-treated with diltiazem showed a reduction in ictal-induced tachycardia at 1 hour whilst post-treatment with diltiazem only reduced the tachycardia seen at the end of the 3 hour ECG recording period. Post-treatment with both atenolol and diltiazem returned SBP to baseline levels. Interestingly, seizure behavioural scores were significantly reduced in atenolol treated rats. Histological investigations also showed that treatment with these cardiovascular drugs reduced apoptotic cell death, inflammatory cell infiltration and the level of structural cardiac damage, including fibrosis and oedema. This finding is of significance as the development of these structural abnormalities in the myocardial tissue can alter cardiac conduction and increase the risk of arrhythmia. Administration of the pro-arrhythmogenic agent aconitine (0.5 mg/kg sc.) was used to show a reduction in the time to arrhythmia onset as a consequence of seizure in KA-saline animals versus naïve saline control. Intervention with both cardiac drugs in the seizure animals negated the significant effect of seizure on cardiac susceptibility to arrhythmia. The current findings suggest that pharmacological intervention with atenolol or diltiazem can provide protection from seizure-induced cardiac injury and therefore should be considered as an adjunct therapy in severe epilepsy.
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Abbreviations

AEDs: anti-epileptic drugs
AMPA: $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANS: autonomic nervous system
BBB: blood brain barrier
BP: blood pressure
CCB: calcium channel blocker
EEG: electroencephalography
ECG: electrocardiography
GABA: $\gamma$-aminobutyric acid
HR: heart rate
KA: kainic acid
ih.: intrahippocampal
MABP: mean arterial blood pressure
NMDA: N-methyl-D-aspartate
PNS: parasympathetic nervous system
PVC: premature ventricular contraction
QTc: QT interval correct for heart rate
sc.: subcutaneous
SBP: systolic blood pressure
SE: status epilepticus
SNS: sympathetic nervous system
SSSE: self-sustained status epilepticus
TLE: temporal lobe epilepsy
VT: ventricular tachyarrhythmia
WDS: wet dog shakes
1 Introduction

1.1 Epilepsy

Epilepsy is a common neurological disorder, affecting approximately 50 million people worldwide (Xie et al., 2011; WHO, 2012). The disorder is characterised by an unprovoked, transient, synchronous pattern of brain activity, otherwise known as a seizure (Blümcke et al., 1999; reviewed by Engelborghs et al., 2000; Fisher et al., 2005). These ictal episodes can range from small muscle-twitches lasting a few minutes to prolonged motor convulsions lasting up to 30 minutes, termed status epilepticus (SE) (Knake et al., 2009). Despite the high prevalence and adverse effects of epilepsy, such as ongoing cognitive disorders, mental health issues and death, it is estimated that 25% of patients suffer from drug resistant epilepsy in which their seizures cannot be controlled by current pharmacological treatments (Jokeit et al., 1999; Sander, 2003; Henning et al., 2011). Epileptics have a mortality rate of two to three times that of the general population, thereby emphasising the need for therapeutic agents that can attenuate seizure-mediated damage (Shackleton et al., 1999; Lhatoo et al., 2001; Morgan et al., 2002).

1.1.1 Pathophysiology

Epilepsy is defined as a group of neurological disorders that are characterised by the development of seizures (Blümcke et al., 1999; reviewed by Engelborghs et al., 2000; Fisher et al., 2005). The onset of these seizures is characterised by a high frequency discharge of action potentials and hyper-synchronisation of a neuronal population. This action potential burst occurs from an influx of extracellular Ca$^{2+}$ causing voltage dependent sodium channels to open. The increase in intracellular Na$^{+}$ levels leads to the generation of multiple action potentials, producing prolonged neuronal membrane depolarisation. This repetitive excitatory discharge occurring within an aggregate of neurons can then spread to surrounding neuronal populations and propagate throughout the brain to cause widespread dysregulation (as reviewed by Engelborghs et al., 2000).

The generation of seizures occurs as a result of increased excitation or decreased inhibition within the brain (as reviewed by Engelborghs et al., 2000). The two primary neurotransmitters within the brain responsible for regulation of neuronal excitation and inhibition are glutamate
and γ-aminobutyric acid (GABA), respectively. An imbalance in the levels and activity of these neurotransmitters and their receptors is suggested as the main factor in seizure progression (as reviewed by Engelborghs et al., 2000). Decreased GABA-mediated inhibition is thought to occur through a variety of mechanisms, including incomplete GABA neuron stimulation, defective GABA$_A$/GABA$_B$ receptor activation and inadequate intracellular calcium buffering. These changes alter the levels of intracellular ions such as Cl$^-$, K$^+$ and Ca$^{2+}$, therefore disrupting normal cell membrane potential. In comparison, mechanisms that have been implicated in development of excess neuronal excitation include, increased N-methyl-D-aspartate (NMDA) receptor activation, upregulated mossy-fibre sprouting leading to recurrent excitatory collateral formation and increased neuronal synchrony due non-synaptic electric field interactions. These modifications cause excess cellular excitation which results in altered phosphorylation and gene expression (as reviewed by Engelborghs et al., 2000).

1.1.2 Aetiology and classification of epilepsy and seizures

Epileptic seizures are classified as idiopathic (primary) or symptomatic (secondary) according to aetiology. The development of symptomatic epileptic seizures has been linked to both environmental (e.g. infection, head trauma, stroke) and genetic (e.g. Fragile X Syndrome) factors (Chapman, 2000; Carriero et al., 2012). These environmental factors can cause structural modifications resulting in irregular ion levels, whilst genetic mutations and metabolic disorders can alter cellular ion levels via neuronal membrane channel dysfunction (Chapman, 2000; Xiao et al., 2004)

The International Classification of Epileptic Seizures officially recognises two broad categories of seizures, focal (partial) and generalised (Berg et al., 2010) (Table 1.). Focal seizures originate within networks limited to one hemisphere. These seizures may be discrete or widespread however, the site of ictal onset within the cortex must be consistent from one seizure episode to the next (Berg et al., 2010). In comparison, generalised epileptic seizures are defined as seizures that develop and quickly spread within bilaterally distributed networks. These seizures can excite multiple regions of the brain but do not have to engage the entire cortex. Additionally, the position and lateralisation of these seizures may not be consistent between ictal episodes (Berg et al., 2010).
Table 1. Seizure classification (adapted from Berg et al., 2010).

<table>
<thead>
<tr>
<th>Generalised seizures</th>
<th>Focal seizures</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonic-clonic</td>
<td>Simple partial</td>
<td>Epileptic spasms</td>
</tr>
<tr>
<td>Absence</td>
<td>Complex partial</td>
<td></td>
</tr>
<tr>
<td>Myoclonic</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.1.3 Mortality

The mortality rate in epileptic individuals is two to three times that of the general population, with patients suffering from chronic, uncontrolled epilepsy reported to have the highest likelihood of premature death (Shackleton et al., 1999; Lhatoo et al., 2001; Morgan et al., 2002; WHO, 2012). Sudden unexpected death in epilepsy (SUDEP) is one of the most common causes of epilepsy-related death and accounts for an estimated 7-17% of deaths in the general epileptic population, and 50% for those with refractory epilepsy (Leestma et al., 1989). Although the exact cause of death is not known in these patients, both cardiac arrhythmias and respiratory failure have been implicated as major contributing factors (Leestma et al., 1989; Nashef et al., 1996; Opeskin et al., 2000). Additionally, other factors such as seizure type, frequency, duration, patient age and the time of day at which seizures occur, have all been found to contribute to the risk of mortality (Leestma et al., 1989; Kloster et al., 1999; Nei et al., 2004).

A review of the literature suggests that pharmacological therapy with anti-epileptic drugs (AEDs) can result in successful management of epilepsy in 70% of newly diagnosed cases, with attenuation of AED treatment often possible after two to five years of therapy (WHO, 2012). This successful management of epilepsy correlates with reduced ictal-induced deaths in the developed world (WHO, 2012). However, close to 80% of epilepsy cases worldwide are found in developing countries where access to AED treatment is limited (Scott et al., 2001; Ding et al., 2006; WHO, 2012). It is this high prevalence of untreated individuals that causes the global rates of ictal-induced mortality to remain high.

1.1.4 Current Pharmacological Therapy with AEDs

There are currently over 20 AEDs that are approved by the FDA for use in the treatment of epilepsy. AEDs primarily work through decreasing the excitatory actions of glutamate,
increasing the inhibitory actions of GABA or modifying ion channel function (reviewed by Kwan et al., 2000; Czapinski et al., 2005). One of the most commonly prescribed classes of AEDs are Na\(^+\) channel blockers (e.g. valproate, carbamazepine and phenytoin) which act to block voltage-gated Na\(^+\) channels and therefore prevent Na\(^+\) influx into the neuron (reviewed by Tunnicliff, 1996; Brodie et al., 1997; Kuo et al., 1997; Kwan et al., 2000; Czapinski et al., 2005). Through a reduction in Na\(^+\) influx, these drugs are understood to help stabilise neuronal membranes, decrease excitatory neurotransmitter release and attenuate the propagation of repetitive action potentials. This decrease in neuronal action potential firing inhibits the spread of excitation within the brain and the development of seizures (Lees et al., 1993; reviewed by Tunnicliff, 1996; Kwan et al., 2000). However, current AEDs also have the potential to cause serious side effects such as cardiac dysfunction, pulmonary disturbances and negative effects on bone (Kasarskis et al., 1992; Yoong et al., 2009; Pack, 2011; Acharya et al., 2013). For example, studies have showed that valproate and vigabatrin are associated with an increased risk of hypertension, dyslipidemia and cardiovascular disease, whilst carbamazepine can decrease bone mineral density (reviewed by Jallon et al., 2001; Pack, 2011; Perucca et al., 2012).

In comparison, GABAergic AEDs, including vigabatrin, attenuate seizure activity by elevating levels of inhibitory GABA within the brain (reviewed by Brodie et al., 1997; Engelborghs et al., 2000; Czapinski et al., 2005). Vigabatrin is an irreversible, enzyme activated GABA transaminase suicide inhibitor used to prevent GABA catabolism (reviewed by Schechter, 1989; Engelborghs et al., 2000; Czapinski et al., 2005). This elevated level of GABA modulates neuronal excitability through an influx of chloride ions or an efflux of potassium ions (Pham et al., 1998; reviewed by Kwan et al., 2000; Czapinski et al., 2005).

Drugs that solely inhibit ionotrophic glutamate receptors within the brain are not commonly used in epileptic patients (reviewed by Kwan et al., 2000). Rather, AEDs that modulate a number of pathways, of which the glutamate system is one, are more frequently seen in the treatment of epilepsy (Upton, 1994; Kwan et al., 2000). For example, felbamate is one such drug that is thought to not only act through inhibition of NMDA receptors but also via modulation of GABA levels (McCabe et al., 1993; Rho et al., 1994; reviewed by Upton, 1994; Czapinski et al., 2005). AEDs have been shown to possess multiple pharmacological properties, and are considered to contribute to the attenuation of seizure through a number of mechanisms. Valproate for example can block voltage gated Na\(^+\) channels as well as increase
the level of GABA within the brain (Rowley et al., 1995; reviewed by Kwan et al., 2000; Czapinski et al., 2005).

### 1.2 Autonomic Nervous System

The autonomic nervous system (ANS) is the primary pathway within the body that is responsible for homeostatic maintenance (reviewed by Shields Jr, 1993; Jansen et al., 2010). It is composed of two subsystems, the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS) (reviewed by Jansen et al., 2010). The control and activation of the ANS is mediated through a complex connection of cortical limbic areas, such as the amygdala and insular cortex, as well as subcortical regions, including the hypothalamus, solitary tract nucleus, ventrolateral medulla and the periaqueductal grey matter (Davis et al., 1993; reviewed by Shields Jr, 1993; Devinsky, 2004; Jansen et al., 2010). These higher brain centres are densely innervated by a series of afferent and efferent nerve fibres therefore creating an elaborate communication network that allows for different cerebral regions to influence one another (Davis et al., 1993; reviewed by Shields Jr, 1993). Differential activation of the SNS and PNS provides the mechanism through which the ANS regulates bodily functions such as heart rate (HR), respiration, digestion, reproduction and excretion (as reviewed by Shields Jr, 1993; Berilgen et al., 2004; as reviewed by Jansen et al., 2010). An imbalance between the sympathetic and parasympathetic input to one or more major organs consequently has the potential to cause serious damage and in some cases death (Metcalf et al., 2009a). This extensive homeostatic influence that the ANS has over the entire body therefore requires tight regulation to prevent the development of physiological irregularities (reviewed by Shields Jr, 1993).

The sympathetic branch of the ANS is responsible for the “fight or flight” response, with activation of this pathway causing increased HR and force of contraction, bronchodilation, enhanced skeletal muscle blood flow and pupillary dilation (reviewed by Shields Jr, 1993; Jansen et al., 2010). These physiological effects of the SNS are primarily mediated by the controlled release of noradrenaline onto adrenergic receptors at the site of action (Norberg, 1967). However, innervation of the adrenal gland by preganglionic sympathetic nerve fibres also allows for the excretion of adrenaline directly into the bloodstream, therefore producing a diffuse and widespread effect (reviewed by Shields Jr, 1993).
In comparison, the PNS is primarily responsible for the regulation of physiological activity when the body is at rest, otherwise known as the ‘rest and digest’ response (reviewed by Shields Jr, 1993). Unlike the SNS, activation of this pathway occurs in a more localised manner and results in the release of the neurotransmitter acetylcholine from postganglionic parasympathetic fibres (Norberg, 1967). This action produces increased gastric motility, decreased HR and force of contraction, salivation, enhanced glandular activity and sphincter relaxation for defecation (reviewed by Shields Jr, 1993). The parasympathetic pathway is therefore crucial for maintenance of the body’s normal physiological function as it allows for the conservation and storage of energy.

1.2.1  Autonomic nervous system and the heart

The ANS is the primary pathway within the body responsible for homeostasis and as a result has a major influence on cardiac function (reviewed by Shields Jr, 1993; Triposkiadis et al., 2009; Jansen et al., 2010). To achieve this level of control, sympathetic neurons from the rostral ventrolateral medulla and parasympathetic neurons from the medulla oblongata, innervate both cardiac tissue and vascular smooth muscle (reviewed by Shields Jr, 1993; Triposkiadis et al., 2009; Jansen et al., 2010). Neuronal fibres from the sympathetic branch of the ANS are present throughout the atria and ventricles of the heart whilst parasympathetic fibres primarily innervate the sinoatrial and atrioventricular nodes via the vagal nerve (Gabella, 2001; reviewed by Devinsky, 2004). This dual innervation of the cardiac tissue allows for each of these pathways to modulate cardiac function, resulting in both chronotropic and ionotrophic (Gabella, 2001; reviewed by Triposkiadis et al., 2009; Jansen et al., 2010).

Constant monitoring and tight control of the sympathovagal balance must occur within the body to prevent the development of cardiac abnormalities such as arrhythmia (Davis et al., 1993; reviewed by Zipes, 2008). The activity of cardiac structures, such as nodal tissue, is therefore under greater influence from the parasympathetic pathway (at rest) in order to reduce the pro-arrhythmogenic effects of the SNS (Levy et al., 1989; as reviewed by Anderson, 2003). In addition to these direct effects from cardiac tissue innervation, the SNS also modulates cardiovascular through stimulation of the adrenal medulla (Anton et al., 1977; reviewed by Shields Jr, 1993; Triposkiadis et al., 2009). The release of acetylcholine from preganglionic nerve fibres, innervating the adrenal medulla, causes the secretion of adrenaline and noradrenaline directly into the blood stream. Once in the blood these hormonal
neurotransmitters can travel to the heart where they interact with peripheral adrenergic receptors (Triposkiadis et al., 2009). This interaction, primarily with the $\beta_1$ adrenergic receptor, causes an increase in HR and contraction force therefore altering the workload of the heart (Anton et al., 1977; reviewed by Shields Jr, 1993; Triposkiadis et al., 2009). Furthermore, cardiac activity can be manipulated through activation of the baroreflex system. This system involves stretch receptors known as the baroreceptors (mechanoreceptors), located in the carotid sinuses, aortic arch vena cava and heart, detecting changes in blood pressure (BP) (Brown, 1980; Shields Jr, 1993). When changes are detected by these neurons, signals are transmitted to the brainstem and a compensatory change in cardiac (HR) and vascular function (constriction/relaxation) occurs to stabilise the BP (Brown, 1980; Shields Jr, 1993). Finally, cardiac activity can also be modulated through activation of the renin angiotensin-aldosterone system (RAAS), which is associated with alterations in fluid retention (Unger et al., 2004; Duprez, 2006).

1.2.2 Electrophysiology of the heart

The primary pacemaker site within the heart is the sinoatrial (SA) node (reviewed by Fleckenstein, 1977; Klabunde, 2011). This structure, located within the posterior wall of the right atrium, is responsible for initiating the wave of atrial depolarisation (Fleckenstein, 1977). However, further conduction of this electrical activity through the ventricles is highly regulated by another group of pacemaker cells known as the atrioventricular (AV) node. These cells slow the impulse, allowing for controlled dispersion (via Purkinje fibres) of the depolarising wave throughout the left and right branches of the ventricle (reviewed by Klabunde, 2011).

In comparison with the atrial and ventricular myocytes, the cells of these two pacemaker nodes possess intrinsic electrical activity that allows for spontaneous depolarisation (reviewed by Klabunde, 2011). The entry of Ca$^{2+}$ (Phase 0) into the pacemaker cells (via L-type Ca$^{2+}$ channels) and Na$^+$ (Phase 0) into nonpacemaker cells results in depolarisation (reviewed by Janvier et al., 1996). Following this impulse, repolarisation of both the pacemaker (Phase 3) and nonpacemaker (Phase 1-3) cells takes place through the efflux of K$^+$ ions (delayed rectifier K$^+$ channels) and efflux/sequestration of Ca$^{2+}$ ions (reviewed by Janvier et al., 1996; Klabunde, 2011).
Figure 1. ECG waves. A. Human ECG trace, B. Rat ECG trace. Adapted from Klabunde (2011).

The ECG is a diagnostic tool that uses surface electrodes to measure population spikes of electrical currents travelling through the heart (Figure 1.). The ECG trace initiates with the P wave, which represents the spread of atrial depolarisation from the SA node (reviewed by Klabunde, 2011). The subsequent QRS complex formation represents ventricular depolarisation activity and is followed by the presentation of the T wave, representing repolarisation of the ventricles (reviewed by Klabunde, 2011). Changes in the amplitude and duration of ECG wave segments are commonly used as markers of cardiac irregularities and will be discussed in relation to ictal presentations below (reviewed by Schwartz et al., 1978; Scott et al., 1985; Klabunde, 2011).

1.3 β-adrenergic receptors

Adrenergic receptors are 7-transmembrane G protein-coupled receptors (GPCR) that regulate a number of physiological functions within the body (reviewed by Marino et al., 2013). These receptors are subdivided into alpha (α) and beta (β) subtypes and are expressed throughout the central nervous system and peripheral tissues. β receptors form the primary adrenergic receptors, expressed in the heart, and therefore mainly responsible for controlling cardiac chronotropic, inotropic and lusitropic responses (reviewed by Lohse et al., 2003; Triposkiadis et al., 2009; Marino et al., 2013). The β₁ and β₂ receptor subtypes are expressed on the heart in a ratio of approximately 70:30, (reviewed by Triposkiadis et al., 2009; Marino et al., 2013). Activation of β₁ receptors predominantly results in enhanced cardiac output through an increase in HR, contractility, automaticity and conduction (reviewed by Marino et al., 2013).

The classical common pathway through which β receptors manipulate cardiac function involves changes in Ca²⁺ handling within the cell (reviewed by Lohse et al., 2003). Ligand
binding of the GPCR causes release of the $G_s$ subunit and subsequent activation of adenylyl cyclase. Stimulation of this enzyme increases the level of cAMP, which can then activate cAMP-dependent protein kinase A (PKA). Active PKA within the myocardial cell phosphorylates a number of essential proteins such as L-type calcium channels, phospholamban and ryanodine receptors. The activation of these essential proteins results in $Ca^{2+}$ influx into the cell and alteration of intracellular $Ca^{2+}$ handling, thereby modifying myocardial contractile activity (reviewed by Lohse et al., 2003).

1.4 Calcium Channels

$Ca^{2+}$ channels are the primary gateway for the entry of $Ca^{2+}$ ions into the cell and therefore are key regulators of intracellular signalling (reviewed by DeWitt et al., 2004; Catterall et al., 2005; Grant, 2009). These voltage-gated channels are embedded within the plasma membrane of electrically excitable cells and for this reason are predominantly located in the ANS, brain, heart and skeletal muscle (reviewed by DeWitt et al., 2004; Catterall et al., 2005). Within the heart there are two primary types of $Ca^{2+}$ channels, low threshold (L) type $Ca^{2+}$ channels, found throughout the heart, and transient (T) type $Ca^{2+}$ channels, which are located in atrial, Purkinje and pacemaker cells (reviewed by Kamp et al., 2000; DeWitt et al., 2004). These channels are formed from various combinations of $\alpha$, $\beta$, $\gamma$ and $\delta$ subunits, with the composition of these multi-subunit complexes determining the function of the channel (Catterall et al., 2005; reviewed by Grant, 2009). The influx of $Ca^{2+}$ that occurs following the opening of these membrane pores allows for the initiation (T-type) and sustainment (L-type) of action potentials. Channel activation is therefore essential for excitation-contraction coupling of skeletal, smooth and cardiac muscle as a well as neurotransmission (reviewed by Braunwald, 1982; reviewed by Kamp et al., 2000; DeWitt et al., 2004; Catterall et al., 2005).

Activation and subsequent opening of L-type $Ca^{2+}$ channels occurs following voltage-gated $Na^+$ channel initiated membrane depolarisation (reviewed by Kamp et al., 2000; Catterall et al., 2005). This influx of $Ca^{2+}$ ions forms a $Ca^{2+}$ spark triggering a further large release of intracellular $Ca^{2+}$ through the activation and opening of the sarcoplasmic reticulum ryanodine-sensitive $Ca^{2+}$ channels. This evoked $Ca^{2+}$ release into the cytoplasm in turn leads to myosin binding and contraction of the cardiomyocyte (reviewed by Braunwald, 1982; DeWitt et al., 2004). In atrial smooth muscle however, contraction requires the binding of
Ca\(^{2+}\) to calmodulin and subsequent activation of myosin light chain kinase (reviewed by Braunwald, 1982; Kamp et al., 2000; DeWitt et al., 2004).

## 1.5 Ictal-induced cardiac irregularities

The spread of seizures throughout the brain can disrupt a number of systems, resulting in a range of seizure-associated symptoms. Excessive neuronal excitability within the central autonomic brain regions has been observed to elicit a range of symptoms of autonomic dysfunction, including cardiovascular and respiratory effects (reviewed by Devinsky, 2004; Jansen et al., 2010). In particular, cardiovascular effects such as, HR alterations, cardiac rhythm dysregulation, hypertension and vasospasms are frequently observed (Blumhardt et al., 1986; Nei et al., 2000; reviewed by Jansen et al., 2010).

The diversity of autonomic cardiovascular changes seen in seizures is dependent on a number of factors including seizure type, frequency, length and patient age (Nei et al., 2000; Opherk et al., 2002; Nei et al., 2004). Variation in these factors can result in differential stimulation of the SNS and PNS, therefore giving rise to the varied cardiac alterations seen between epileptic patients (Blumhardt et al., 1986; Tinuper et al., 2001; Opherk et al., 2002; as reviewed by Jansen et al., 2010). This dissimilarity in ictal-induced cardiac alterations is evident with patient HRs, as some individuals present with ictal-induced bradycardia whilst others display evidence of ictal-induced tachycardia (Tinuper et al., 2001; Opherk et al., 2002; Leutmezer et al., 2003; Rugg-Gunn et al., 2004; as reviewed by Jansen et al., 2010). Previous research has shown this tachycardic development commonly occurs in patients with generalised and right-sided temporal seizures, whilst bradycardia is more frequently seen in seizures of the left hemisphere (Nei et al., 2000; Opherk et al., 2002; as reviewed by Jansen et al., 2010). These findings highlight the diverse autonomic changes and cardiac alterations that can occur as a result of seizure duration, frequency and location within the brain.

### 1.5.1 Tachycardia

Seizure duration and frequency have been closely linked with the development of cardiac alterations (Nei et al., 2000; Metcalf et al., 2009a; Read et al., 2014a). Studies examining electrocardiography (ECG) changes in human epileptic patients consistently report increase in HR as the predominant cardiac alteration displayed (Opherk et al., 2002; Leutmezer et al.,
2003). Opherk et al. (2002) showed 100% of individuals with generalised seizures had an ictal or post-ictal increase in HR. In conjunction with these results, Zijlmans et al. (2002) found an increase of more than 10 beats per minute (b.p.m.) occurred in 73% of seizures (93% of patients) and greater than 20 b.p.m. in 55% of seizures (80% of patients).

Tachycardia (HR>100 b.p.m.) is frequently observed in clinical studies of epilepsy, with Keilson et al., (1989) showing the development of tachycardia in 93% of seizures (Keilson et al., 1989). Development of tachycardia has been suggested to occur as a consequence of a shift in the sympathovagal balance during seizure, resulting in enhanced sympathetic activity and/or decreased parasympathetic dominance (Faustmann et al., 1994; Druschky et al., 2001; Hilz et al., 2002; reviewed by Devinsky, 2004; Jansen et al., 2010).

The pronounced periods of tachycardia that occur from seizure-induced autonomic imbalance and repetitive cardiac stimulation promote the development of arrhythmias (Nei et al., 2000; Opherk et al., 2002; Borowicz et al., 2014). This increased risk of arrhythmia can occur from direct electrical disruption and/or structural damage to the heart (Opherk et al., 2002). The impaired vagal activity and increased sympathetic drive that is associated with epilepsy can prevent the restoration of normal HR following seizure-induced tachycardia (Damasceno et al., 2013; Borowicz et al., 2014). This disturbance in the ability of the heart to return to baseline levels therefore promotes the development of abnormal cardiac conduction (Borowicz et al., 2014). Additionally, the rapid rise in HR seen during seizures, significantly increases the cardiac workload and myocardial oxygen demand (Heusch et al., 2007). This increased strain on the heart can promote the development of transient myocardial structural injury, such as ischaemic microinfarcts, and thereby facilitate the generation of lethal arrhythmias (Chen et al., 2002; Coronel et al., 2005; Heusch et al., 2007; Borowicz et al., 2014; Read et al., 2014a).

1.5.2 ECG abnormalities

Ictal-induced ECG abnormalities, other than tachycardia, are commonly seen in epilepsy, with reports of irregularities presenting in 31% of seizures and 72% of epileptics (Opherk et al., 2002; reviewed by Devinsky, 2004; Surges et al., 2009). Abnormalities that frequently present in epileptic patients include, prolonged QT interval (ventricular depolarisation and repolarisation interval), premature ventricular depolarisation and atrial fibrillation (Nei et al., 2000; Opherk et al., 2002; Zijlmans et al., 2002). These changes can be sub-symptomatic but
may lead to cardiac injury resulting in the development serious ECG abnormalities such as ST-segment depression and T-wave inversion, with these occurring in 10% of patients (Opherk et al., 2002). The development of these ECG irregularities can occur as a result of structural damage developing consequent to intense repetitive autonomic stimulation or excessive stimulation directly to the heart (Opherk et al., 2002). The tissue damage that can occur from repetitive stimulation often involves impairment of cardiac ion channels or formation of fibrotic microlesions which disrupt the cardiac depolarising wave (Opherk et al., 2002; Surges et al., 2009; Engelman et al., 2010). The extent of ECG abnormality development has been correlated with the duration and frequency of the seizure episodes (Nei et al., 2004). It is therefore important to closely monitor those patients suffering frequent seizure activity in order to reduce the risk of life-threatening arrhythmias such as ventricular fibrillation developing.

1.5.3 Vascular Tone

The widespread sympathetic surge that occurs during seizures can affect vascular tissues and result in the development of vasospasms that cause fluctuations in tissue perfusion and in BP. (Natelson et al., 1998; reviewed by Surges et al., 2009). As arteries, arterioles and veins, are highly innervated by postganglionic sympathetic nerves, the intense sympathetic discharge that is associated with seizures is capable of changing vascular tone (Surges et al., 2009; Thomas, 2011). In addition, the high level of circulating catecholamines interacts with the vasculature causing uncontrolled contraction (Heros et al., 1983; Natelson et al., 1998; Sakuragi et al., 2007; Lyon et al., 2008). The majority of studies examining changes in this parameter have found a significant increase in mean arterial blood pressure (MABP) during seizures however, it must be noted that cases of decreased or no change in BP have also been seen (van Buren, 1958; Lou et al., 1979; Surges et al., 2009). Furthermore, these changes in BP have been seen to occur at both ictal and post-ictal time points. A study by Lou and colleagues (1979) looking at MABP during epileptic seizures in the newborn, found that MABP increased by 100% during generalised tonic-clonic convulsions. Furthermore, the authors stated that this increase in MABP occurred very rapidly at the onset of seizure and was likely due to increased sympathetic tone (Lou et al., 1979). Prior support of these findings comes from an early study examining autonomic changes during seizures in patients with temporal lobe epilepsy (TLE) (van Buren, 1958). A significant hypertensive response with elevations in both diastolic and systolic BP (SBP) was seen to occur at the time of
seizure. The consequent elevated pressure gradient will eventually result in greater compensatory stroke volume commonly associated with the development of an increase in ventricular wall stress or afterload (Little et al., 1982). This increased afterload has been shown to contribute to the development of cardiac hypertrophy and myocardial stunning (Stahl et al., 1986).

In addition to a rise in BP, studies have also found evidence of impaired baroreflex sensitivity in epileptic patients (Dütsch et al., 2006). For example, a study by Dütsch et al., (2006) showed that patients with drug refractory TLE had decreased cardiovascular variability and a reduction or resetting in baroreflex sensitivity. This decrease in sensitivity can be extremely serious as the baroreflex system allows the body to detect and compensate for changes in BP by modulating cardiac function and vascular activity (Lanfranchi et al., 2002). A decrease in this sensitivity inhibits the physiological response to changes, such as the hypertensive state seen during seizures, and therefore can promote further damage to structures such as the heart (van Buren, 1958; Lanfranchi et al., 2002; Dütsch et al., 2006). This increase in BP, along with impaired baroreflex sensitivity, may therefore form important factors in the development of seizure-related death.

1.5.4 Cardiac Structural damage

The intense neuronal excitability that occurs in the brain during seizures causes profound stimulation of the SNS and subsequent catecholamine release (Simon et al., 1984; Sakuragi et al., 2007). This pathophysiological disruption can alter myocardial function, resulting in lethal ischaemic damage and cardiomyocyte degeneration (Chen et al., 2002; Simona et al., 2005; Metcalf et al., 2009b; Bealer et al., 2010; Read et al., 2014a). A study by Read et al. (2014) showed kainic acid (KA)-induced seizure rats with pronounced tachycardia had significantly higher levels of infarct damage and interstitial fibrosis as early as 48 hours post-seizure induction, when compared with control rats. This injury may have resulted from the decreased cardiac perfusion occurring during tachycardia. Clinical evidence supporting these findings was produced by Natelson et al., (1998), who found the presence of both interstitial and perivascular fibrosis in the hearts of epileptic patients who died suddenly. The authors also suggested that the development of these fibrotic regions may occur from a reduction in cardiac oxygen supply as a result of seizure-induced coronary vasospasms (Natelson et al., 1998). Furthermore, a study by Simona et al. (2005) looking at post-mortem myocardial
sections of SUDEP patients also found evidence of several fibrotic foci in the subendocardial myocardium in 40% of patients examined. Formation of these microinfarcts has the capacity to disrupt myocardial depolarisation, allowing for re-entrant excitation and therefore the development of potentially lethal cardiac arrhythmias and heart block (Nei et al., 2000; Coronel et al., 2005; as reviewed by Surges et al., 2009).

In addition to the development of ischaemic damage, research has shown evidence of increased cardiomyocyte death following seizure. A study by Metcalf et al. (2009b) showed SE rats had significantly higher post-ictal concentrations of cardiac troponin I in the plasma, when compared to control. In support of this finding, a case report by Park et al. (2011) found elevated cardiac troponin levels in an epileptic individual following an episode of generalised tonic-clonic seizures. Furthermore, Hajsadeghi et al. (2009) found a significant correlation between the serum level of cardiac troponin I and the number of seizures. These findings of increased plasma levels of troponin I, a highly specific myofibrillar protein, represent the occurrence of ictal-induced cardiomyocyte death in the heart (O'brien et al., 2006; Hajsadeghi et al., 2009; Bealer et al., 2010). It must also be noted that this cardiac damage can result in the initiation of an inflammatory response (Frangogiannis, 2006b; Frangogiannis, 2006a). This response commonly occurs due to cytokine release from necrotic cells in an ischaemic area of the heart. These cytokines are involved in the recruitment of circulating cells such as macrophages, which play an important role in removing debris and promoting tissue repair (Frangogiannis, 2006a; Fischer et al., 2007). Although this initial inflammatory response is necessary for healing and repair, chronic activation can result in further damage (Fischer et al., 2007).

1.6 Models of epilepsy

The use of relevant, reproducible animal models of epilepsy is critical to the development of new improved pharmacotherapies. Animal models currently used for experimental research into epilepsy can be separated into two distinct categories of genetic or acquired epilepsy (reviewed by Löscher, 2002; Frangogiannis, 2006a).

Genetic (idiopathic) animal models are commonly used in the study of epilepsy, with the majority of these animals displaying generalised or absence seizures (Gower et al., 1995; reviewed by Prasad et al., 1999; Löscher, 2002). This model can be subdivided into two categories of spontaneous or induced mutations. Spontaneous mutation models are more
commonly used than transgenic or knockout animals in the development of new pharmacotherapies. One of the primary reasons for this preference is the ability of the spontaneous mutation models to produce seizure responses to specific sensory stimuli, otherwise known as reflex epilepsy (reviewed by Prasad et al., 1999; Löscher, 2002). The use of these genetic models has consequently provided valuable insight into the pathophysiological alterations resulting from chronic seizures experienced in epilepsy.

Acquired (symptomatic) models of epilepsy involve the use of either chemical or electrical stimulation to induce seizures. Electrical seizure models involve exposing brain regions, such as the hippocampus or amygdala, to short periods of electrical stimulation (Goddard et al., 1969; reviewed by Sato et al., 1990; Löscher, 2011). Chemical stimulation requires exposure of the animal to neuroexcitatory agents, such as KA or domoic acid (structural glutamate analogues) and pilocarpine (muscarinic receptor agonist), which induce transient, synchronous patterns of neuronal activation (French et al., 1982; reviewed by Löscher, 2002; Löscher, 2011; Vranyac-Tramoundanas et al., 2011; Read et al., 2014a). The use of these models is commonly seen in epilepsy research and has therefore led to further subdivision of these seizure induction methods into kindling and self-sustained status epilepticus (SSSE) models (reviewed by Sato et al., 1990; Löscher, 2002; Löscher, 2011). The kindling model involves exposing limbic brain regions of healthy animals to short repeated burst of electrical or chemical stimulation (reviewed by Sato et al., 1990; Löscher, 2011). The use of this technique results in a gradual increase in neuronal and behavioural seizure activity, with the subsequent development of elicited seizures, similar to those that present in patients with TLE (reviewed by Sato et al., 1990; Löscher, 2011). Alternatively, SSSE models involve sustained electrical or chemical stimulation of limbic regions, such as the hippocampus (reviewed by Lemos et al., 1995; Goodman, 1998; Löscher, 2002). The use of this model results in the development of focal seizures that progress on to secondary generalised seizures, with recurrent spontaneous seizures occurring over time (reviewed by Goodman, 1998; Löscher, 2002; Löscher, 2011).

The type of chemoconvulsant and route of administration used in animal models of epilepsy is extremely important, as different models have been seen to produce varying degrees of neuronal excitability, seizure behaviours and adverse effects, such as mortality rates (as reviewed by Sperk, 1994; Babb et al., 1995; Löscher, 2002). An extensive variation in induction protocol and evoked responses has been reported between the different animal models of epilepsy. Consequently, when examining a therapeutic response, it is crucially
important to select a specific chemoconvulsant and route of administration which is not associated with confounding factors that could potentially impact the results. For example, while commonly used to induce seizures (in conjunction with Li\(^+\)), the muscarinic agonist, pilocarpine, has been found to modulate the actions of cardiac K\(^+\) channels and therefore has the potential to alter HR, excitation conduction and cardiac repolarisation (Wang et al., 1999). In addition to these findings, studies have shown that systemic administration of this excitotoxin is associated with a high mortality (Löschter, 2002). It is therefore evident that the use of certain chemoconvulsants, such as pilocarpine, in the study of epilepsy has the potential to confound experimental findings, in particular those studies looking at seizure-induced cardiac damage.

The ability of systemically administered chemoconvulsants to act peripherally and potentially produce unwanted adverse effects provides justification for the use of other administration routes. The exploitation of intra-cerebral delivery systems, such as intrahippocampal (ih.) drug cannulas, allows for small, localised volumes of the neuroexcitatory agent to be administered directly into the brain. Delivery of such small drug volumes (1-2 µl range), into the hippocampus can significantly reduces the biodistribution of these chemoconvulsants (Vranyac-Tramoundanas et al., 2011). Earlier work by our group has shown that ih. domoic acid administration failed to release detectable levels of the neurotoxin into the systemic circulation (Vranyac-Tramoundanas et al., 2011). This restriction of the chemoconvulsant distribution within specific brain regions ensures that any peripheral effects of the drug stimulus are due to the seizure itself and not the excitotoxin. Previous work carried out in our laboratory has shown that systemic KA administration produced bradycardia within the first 30 minutes, whilst ih. KA administration resulted in tachycardia (Read, 2014; Read et al., 2014a). This disparity in HR following different routes of KA administration suggests a possible peripheral action of the chemoconvulsant on vagal activity. It is for these reasons that animal models, such as ih. KA, are superior when studying the pathological effects of seizure on heart. Furthermore, research carried out in our laboratory has shown that the ih. excitotoxins, domoic acid and KA, induce seizures that result in the development of a cardiomyopathy, demonstrating features of systolic and diastolic dysfunction as well as cardiac injury (Read, 2014; Vranyac-Tramoundanas et al., 2011).
1.6.1 Kainic acid

The excitotoxin, KA, a structural analogue of glutamic acid, is 30-fold more potent than glutamate (as reviewed by Coyle, 1983; Raedt et al., 2009; Tan et al., 2009). Originally isolated from the seaweed Digenea simplex, this compound is now commonly used as a neuroexcitatory amino acid for the induction of seizures in animal models of epilepsy (as reviewed by Coyle, 1983; Raedt et al., 2009). KA acts through binding of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, both of which are subtypes of the ionotropic glutamate receptor (Gluck et al., 2000; Chen et al., 2002; Lee et al., 2008). The resulting increase in intracellular Ca\(^{2+}\) and Na\(^+\) levels as well as upregulated reactive oxygen species production can cause extensive neuronal excitation resulting in ictal-induced neurodegenerative cell death (Schinder et al., 1996; Gluck et al., 2000; Chen et al., 2002). This damage to neuronal structures has been found to occur primarily in the limbic brain structures, with hippocampal CA1 and CA3 region cell damage and death frequently seen (French et al., 1982; Lee et al., 2008). The wide-spread excitatory effects seen following KA administration provide support for the use of this chemoconvulsant agent in the induction of seizures (Gluck et al., 2000).

1.6.2 Temporal lobe epilepsy model

Of the 50 million people worldwide who suffer from epilepsy, around 30% of these individuals are characterised as having TLE (Xie et al., 2011; WHO, 2012). The development of TLE is associated with the development of recurrent complex partial seizures that can progress on to more severe secondary generalised seizures, as the disease worsens (reviewed by Engelborghs et al., 2000). This recurrent neuronal excitability causes neuropathological modifications that can result in cognitive decline (Tasch et al., 1999; Wu et al., 2008; Cersósimo et al., 2011). These changes within the brain include hippocampal circuit restructuring, neurodegeneration and hippocampal sclerosis, all of which can contribute to decreased hippocampal volume (Tasch et al., 1999; Cersósimo et al., 2011). The extensive neuronal damage and high incidence rate that is associated with this form of epilepsy highlights the need for a comparable seizure model that can recreate the pathophysiological seizure activity of TLE (Wu et al., 2008).

Studies have found the use of chemical seizure models, such as KA and domoic acid, as well as electrical hippocampal stimulation result in seizure activity and neuronal damage similar to
that seen in TLE (Babb *et al.*, 1995; Bouilleret *et al.*, 1999; Nissinen *et al.*, 2000; Wu *et al.*, 2008; Raedt *et al.*, 2009; Vranyac-Tramoundanas *et al.*, 2011). The ability of these chemoconvulsants to induce neuronal hyper-excitability primarily within the temporal lobe is thought to occur as a result of the high AMPA and kainate receptor expression in the hippocampus (Monaghan *et al.*, 1982a; Hampson *et al.*, 1992; Malva *et al.*, 1998; Bloss *et al.*, 2010). In particular, the development of a model using ih. KA administration has provided a promising method of inducing seizures that present with the behavioural and pathological features seen in TLE (Babb *et al.*, 1995; Bouilleret *et al.*, 1999; Wu *et al.*, 2008; Raedt *et al.*, 2009). A study by Bouilleret (1999) found ih. administration of KA caused mice to develop recurrent partial seizures and hippocampal sclerosis, similar to that seen in human mesial TLE. In addition, Babb and colleagues (1995) found that rats administered with ih. KA developed ipsilateral interictal spikes, complex partial seizures and hippocampal mossy fiber synaptic reorganisation similar to that seen following hippocampal sclerosis in human TLE. Furthermore, previous work conducted in our lab demonstrated that ih. KA administration in rats resulted in the generation of intermittent seizure activity representative of TLE with secondarily generalised tonic-clonic seizures (Millen 2014, Read 2014). These studies provide evidence to support the use of the ih. KA administration protocol in the rat as a promising model of TLE.

### 1.7 Potential cardioprotective agents

#### 1.7.1 β-blockers

β adrenergic blocking agents or β blockers are a class of pharmacological agents that specifically target and antagonise the β receptor (Heng *et al.*, 1985; reviewed by Frishman, 2008; Frishman *et al.*, 2011). β receptors are widely distributed throughout the body, including the heart, airways, arteries, smooth muscle and kidneys (reviewed by Mason *et al.*, 2009; Nikolaev *et al.*, 2010; Marino *et al.*, 2013). Atenolol is classed as a relatively selective β₁-adrenoceptor antagonist, competing with sympathomimetic neurotransmitters for β₁ receptor binding in the myocardium and nodal tissue (Figure 2.) (reviewed by Frishman, 2008; Frishman *et al.*, 2011). The negative inotropic and chronotropic action of atenolol acts to protect the heart by decreasing both myocardial workload and oxygen consumption (reviewed by Cruickshank *et al.*, 1991; Frishman, 2008; Frishman *et al.*, 2011). The ability of atenolol to selectively block only the β₁ receptor within therapeutic concentrations has been
suggested to avoid the development of adverse side effects, such as bronchial constriction, associated with the non-selective, lipophilic β blockers (reviewed by Mason et al., 2009; Frishman et al., 2011). These beneficial therapeutic effects provide support for the current use of atenolol in the treatment of patients with hypertension, angina, dysrhythmias or myocardial infarction (reviewed by Heng et al., 1985; Frishman, 2008; Mason et al., 2009).

![Chemical structures of atenolol and diltiazem](image)

**Atenolol**

**Diltiazem**

**Figure 2.** Representative chemical structure of atenolol and diltiazem (adapted from Frishman (2008) and Chaffman et al. (1985)).

### 1.7.2 Calcium Channel Blockers

Ca$^{2+}$ channel blockers (CCB) are a class of drugs, which antagonise the influx of Ca$^{2+}$ ions through voltage-gated Ca$^{2+}$ channels in the membrane of cells (reviewed by Braunwald, 1982; Hockerman et al., 1997; Abernethy et al., 1999; DeWitt et al., 2004). Drugs classified as CCBs can be further divided into three subcategories; dihydropyridines, benzothiazepines and phenylalkylamines, based on their distinct chemical structure and effects (reviewed by Hockerman et al., 1997; DeWitt et al., 2004). The administration of these CCBs is now widely accepted for the treatment of cardiovascular disturbances such as hypertension, angina, and arrhythmias (reviewed by Abernethy et al., 1999; DeWitt et al., 2004). However, it must be noted that the class of CCB used is highly dependent on the clinical disorder of the patient as there are differences in tissue selectivity. For example, phenylalkylamines and benzothiazepines are relatively selective for myocardial tissue and are therefore primarily used for the treatment of angina. By comparison, the dihydropyridine, nifedipine, has high
vascular selectivity and therefore is not used in this condition due to the risk of causing reflex tachycardia (reviewed by Abernethy et al., 1999; DeWitt et al., 2004).

Diltiazem is a Ca\(^{2+}\) antagonist from the benzothiazepine class of CCBs (Figure 2.) (reviewed by Hockerman et al., 1997; DeWitt et al., 2004). This drug is considered to be an intermediate between the other two classes of CCBs as it has both cardiac depressant and vasodilatory properties. Administration of diltiazem has been found to produce a decrease in SBP, dilation of coronary arteries and inhibition of spontaneous coronary vasospasms (reviewed by Abernethy et al., 1999; DeWitt et al., 2004). This decrease in cardiac afterload and dilation of the coronary arteries results in reduced cardiac workload and increased myocardial oxygen supply. Furthermore, treatment with diltiazem is also seen to slow conduction through the AV node (reviewed by DeWitt et al., 2004). This action lowers the risk of arrhythmia (Class IV anti-arrhythmic) whilst also decreasing cardiac workload through a reduction in HR (reviewed by Hockerman et al., 1997; DeWitt et al., 2004).

1.8 Rationale

Cardiac dysfunction and structural damage have been frequently associated with seizure activity in both animal and clinical studies. These findings provide support for the use of cardioprotective pharmacological agents during seizure. This study considers two possible treatment scenarios. While prophylactic drug therapy may be applicable in epileptic individuals at high risk of frequent or severe seizure, the use of intervention therapies as an emergency measure should be considered in the acute management of patients presenting with high level prolonged seizures. Both atenolol and diltiazem are used clinically as rate control anti-arrhythmic agents and have been indicated for use in a variety of cardiac pathologies. The value and comparative benefits of these two pharmacological agents are tested in the current study.
1.9 Aim and Hypothesis

The research conducted as part of this thesis aimed to investigate whether pre-treatment and post-treatment with atenolol or diltiazem can protect the heart against seizure-induced cardiac damage in a rat model of epilepsy. It was hypothesised that ih. administration would restrict the KA delivery to the hippocampus to induce seizure activity associated with significant structural and functional cardiac damage. It was further hypothesised that treatment with atenolol or diltiazem would attenuate this seizure-mediated autonomic discharge and therefore protect the heart. The following aims were used to test these hypotheses. The aims sought to confirm the:

- development of seizure behavioural activity in the ih. KA administration model using a modified Raccine seizure scale.
- presence of functional cardiac damage through ECG analysis, with specific focus on HR, QTc interval, T wave amplitude.
- presence of cardiac markers of structural injury, including fibrosis, apoptotic cell death and inflammatory cell infiltration.
- formation of an increased arrhythmogenic risk to seizure.

These aims were tested in the presence and absence of the therapeutic agents, atenolol and diltiazem.
Pre-treatment with Atenolol and Diltiazem
2 Methods

2.1 Materials and Reagents

KA was purchased in powder form from Tocris (Bristol, UK) and dissolved in sterile saline (0.9% NaCl). Atenolol and diltiazem were purchased from Sigma-Aldrich (Auckland, New Zealand). All restricted veterinary medicines used in the surgical procedures and maintenance of the experimental animals were purchased from the University of Otago, Animal Welfare Office (Dunedin, New Zealand). Intrahippocampal drug cannulae (26G) with screw-top caps were obtained to order from Coherent Scientific (Australia). All other materials and reagents were obtained from BDH (Palmerston North, New Zealand) and Sigma-Aldrich (Auckland, New Zealand).

2.2 Animals

Male Sprague Dawley rats (320-350 g) were obtained from the Animal Resource Unit (University of Otago, Dunedin, New Zealand). Animals were acclimated in the Department of Pharmacology and Toxicology animal holding room for 5 days prior to surgery. This room was maintained at 22°C on a regular day-night light cycle (12 h), with animals allowed access to water and standard rat chow ad libitum. Experiments were carried out under consent from University of Otago Animal Ethics Committee and in accordance with the guidelines stated in the University of Otago publication on the Care and Use of Laboratory Animals.

2.3 Surgical Implantation

2.3.1 Transmitter Implantation

Animals were implanted with telemetric transmitters to allow for simultaneous ECG and electroencephalography (EEG) recordings. Transmitters were sterilised by soaking in hibitane (5% chlorhexidine acetate/95% ethanol) for 1 hr prior to surgery. All surgical apparatus and drapes were also sterilised in hibitane for 1 hr prior to surgery.

Animals were administered the antibiotic, amphotrem (60 mg/kg, 0.2 ml, bid., subcutaneous [sc.]), and the non-steroidal anti-inflammatory, carprofen (5 mg/kg, sc.), prior to the initiation of surgery and for the following 3 days. Animals were anaesthetised by the administration of ketamine hydrochloride (75 mg/kg, sc.) and domitor (medetomidine hydrochloride, 0.5 mg/kg,
sc.). Atropine (0.05 mg/kg, sc.) was also given to reduce bronchial mucous secretion and prevent bradycardias, during surgical anaesthesia. Once the pedal withdrawal reflex had been abolished, the eye ointment, Tricin, was applied to prevent the eyes from drying out and sustaining damage. In addition, the throat, scalp and xiphoid region of the abdomen were shaved and disinfected with hibitane in order to comply with aseptic protocols.

Following surgical preparation, animals were placed on a 37°C homoeothermic pad (Harvard Apparatus, Holliston, USA), to maintain body temperature, and covered with a sterilised plastic drape exposing surgical sites. Implantation of surgical transmitters was carried out as previously described (Goulton et al., 2010; Sawant et al., 2010; Read et al., 2014a). A 3 cm incision was made along the skin overlying the lower abdomen and a small subcutaneous pocket created to allow for insertion of a two-channel TR40BB digital telemetric transmitter (Telemetry Research, Auckland, New Zealand). The xiphoid process was exposed and the reference ECG electrode sutured to the dorsal surface. Following this, a 1 cm incision was made at the level of the thorax and a trochar used to tunnel the recording lead, along with two EEG leads, subcutaneously to the rostral thorax. A running subcutaneous suture (5-0 prolene suture, Ethicon, USA) was used to close the abdominal cavity. The trachea was then exposed to allow the ECG recording electrode to be pushed under the muscle, along the trachea and into the anterior mediastinum and sutured to the muscle layer in proximity to the right atrium. The wound was then closed using the same subcutaneous suture technique. A 1 cm incision was made posterior to the foramen magnum, at the back of the neck, and the two EEG leads were exteriorised prior to positioning using the stereotaxic frame.

2.3.2 Cannula Implantation

Animals were placed and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, USA) (Figure 3.) to allow for accurate placement of the 26G ih. drug cannula. Under stereotaxic control, three holes were drilled (1 mm ventral to dura) to allow for implantation of the ih. cannula (5.2 mm posterior to bregma, 5 mm left of midline, 4.2 mm ventral to dura), the EEG recording electrode (5.2 mm posterior to bregma, 5 mm left of midline, 2 mm
ventral to dura) and the EEG reference point electrode (2 mm anterior to bregma, 2 mm right of midline). Dental acrylic was then applied to anchor the cannula to the skull. Once set, the animal was removed from the stereotaxic frame and the head closed with standard surgical suture.

Following completion of the surgery, animals were administered a saline bolus (5 ml, s.c.) and antisedan (atipamezole hydrochloride, 5 mg/ml, s.c.) was given to reverse the effects of domitor, therefore improving recovery. Animals were then left to recover for 7 days prior to experiments.

2.4 Seizure induction and drug treatment

2.4.1 Seizure induction

Seizures were induced at the 7 day post-surgical time point. KA was administered directly into the hippocampus using the ih. cannula port. KA delivery was achieved using a Hamilton glass syringe (Hamilton, Reno, USA) mounted in a Bee Hive microinfusion pump (Bioanalytical Systems, West Lafayette, USA). The use of this system allowed for a single ih. infusion of KA (2 nmol in 1 µl saline) at a rate of 1 µl/min. The dose and infusion rate were determined from research of the literature and previous dose studies in our lab conducted by PhD student Morgayn Read (Dakshinamurti et al., 1991; Sawant et al., 2010; Read, 2014). An equivalent volume (1 µl) of saline (0.9% NaCl, ih.) was given to the non-seizure control rats.

2.4.2 Pre-treatment dosing protocol

Animals were randomly assigned to one of the four different groups ($n = 4$ animals per group). KA seizure animals were treated with saline sc. (saline-KA), 5 mg/kg atenolol sc. (Atenolol-KA) or 2.5 mg/kg bid. diltiazem sc. (diltiazem-KA) and compared to control (non-seizure) group receiving an equivalent volume of saline-vehicle. Rats were pre-treated with the drugs for 3 days prior to seizure induction, with the last pre-seizure dose given 1 h before ih. KA or saline administration. This therapeutic treatment continued daily until animals were euthanised at 48 hr post-seizure induction. Control rats received ih. saline infusion and daily sc. saline injections. The dose of atenolol was derived from previous studies and a human-to-animal dose conversion carried out by PhD student Morgayn Read (Read, 2014). The dose of
diltiazem was determined using a human-to-animal dose conversion calculation to account for the difference in body surface area (Reagan-Shaw et al., 2008).

2.5 Behavioural monitoring

Animal behaviours in the current study were monitored in a custom-made Perspex™ observation chamber (Aburn Glass, Dunedin, New Zealand) (Figure 4.). The animal was placed in the behavioural chamber for 20 min prior to baseline recording to allow for acclimatisation. The use of a Powerlab 2/25 signal conditioner and LabChart v.6 software (ADInstruments, Dunedin, New Zealand) allowed for ECG and behavioural data to be recorded simultaneously.

Prior to seizure induction, a 30 min baseline period was recorded to obtain the resting parameters in each animal. Following ih. KA or saline administration, the rat was immediately returned to the observation chamber and the behavioural and EEG/ECG recording started for a 3 hr monitoring period. Behavioural scores were coded every 15 sec, with distinct behaviours additionally noted. A 5-point ranking scale (adapted from Racine, 1972; Hesp et al., 2007) was used to separate and record behaviours, ranging from level 0 denoting normal behaviours, to level 5 representing clonic-tonic convulsions (Table 1). Behavioural data was also recorded for 60 min at 24 hr, 48 hr and 7 days post-KA administration. Behavioural data was then analysed by taking the highest recorded behavioural score from each minute during the recording period. In addition, the total number of wet dog shakes (WDS) per minute was also recorded.

2.6 Electrocardiography Analysis

Telemetric signals were collected using TR102 dual channel receiver and recorded through a 4-channel PowerLab (ADInstruments, Dunedin, New Zealand). ECG data was sampled using the LabChart v.6 Pro ECG Analysis software (ADInstruments, Dunedin, New Zealand).
Measurements were taken across the 30 min baseline, 3 h recording period and subsequent 60 min recordings at the 24 hr, 48 hr and 7 day time points. Every five minutes of the ECG trace was averaged and analysed to examine changes in HR, T wave amplitude and corrected QT interval (QTc). The use of the Mitchell analysis algorithm allowed for QT intervals to be corrected for changes in HR, \[ QT_c = \frac{QT}{\sqrt{RR/100}} \] (Mitchell et al., 1998). This algorithm corrects for the higher HR and altered ECG wave morphology seen in rodents.

**Table 2.** Seizure behaviour scores (adapted from Hesp et al., 2007).

<table>
<thead>
<tr>
<th>Level 0: Normal behaviours</th>
<th>Level 1: Unusual behaviours</th>
<th>Level 2: Mild seizure behaviours associated with the head and neck</th>
<th>Level 3: Moderate seizure behaviours associated with the limb and trunk</th>
<th>Level 4: Severe seizure behaviours</th>
<th>Level 5: Clonic-tonic convulsions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sleeping</td>
<td>Blinking</td>
<td>Head bobbing</td>
<td>Wet dog shakes</td>
<td>Whole body twitches</td>
<td>Whole body twitches</td>
</tr>
<tr>
<td>Wall climbing</td>
<td>Frozen</td>
<td>Head shakes</td>
<td>Forelimb clonus</td>
<td>Myoclonic jerks</td>
<td>Myoclonic jerks</td>
</tr>
<tr>
<td>Air sniffing</td>
<td>Squinting</td>
<td></td>
<td>Running</td>
<td>Foaming</td>
<td>Foaming</td>
</tr>
<tr>
<td>Face washing</td>
<td>Mastication</td>
<td></td>
<td>Circling</td>
<td>Rigidity</td>
<td>Rigidity</td>
</tr>
<tr>
<td>Resting</td>
<td>Body scrunching</td>
<td></td>
<td>Forelimb tremors</td>
<td>Loss of balance</td>
<td>Loss of balance</td>
</tr>
<tr>
<td>Grooming</td>
<td>Hiccups</td>
<td></td>
<td>Hindlimb twitches</td>
<td>Rearing</td>
<td>Rearing</td>
</tr>
<tr>
<td>Walking</td>
<td>Resting unnaturally</td>
<td></td>
<td>Hindlimb clonus</td>
<td>Tail whipping</td>
<td>Tail whipping</td>
</tr>
<tr>
<td>Snuffling</td>
<td>Panting</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.7 **Statistics**

All statistical analysis of data was carried out using Prism v.5 (GraphPad, San Diego, USA). Behavioural data was analysed using a non-parametric Kruskal-Wallis test. Differences in ECG parameters across various treatment groups were determined through the use of a two-way repeated measures analysis of variance (ANOVA). A Bonferroni post-hoc analysis was used to calculate whether these differences between groups were statistically significant. Statistical significance was determined as \( P < 0.05 \). Data was presented as the mean ± standard error of the mean (SEM).
3 Results

3.1 Behavioural Data

Data recorded in the saline pre-treatment control rats showed that behavioural scores (Table 2.) remained consistently low throughout the study period, with these animals displaying normal behaviours, resulting in a cumulative seizure score of 17 ± 7. Intrahippocampal KA infusion immediately induced high-level seizure behaviours, which remained significantly elevated for the duration of the 180 minute recording period (Figure 5.). Both cumulative seizure score (426 ± 6 vs. 27 ± 7, \( P<0.05 \)) and total WDS (351 ± 51 vs. 3 ± 2, \( P<0.05 \)) were elevated compared to the non-seizure saline control group. Seizure behaviours returned to control levels by 24 hours post-KA. Pre-treatment with atenolol and diltiazem had no significant effect on behavioural scores compared to the saline+KA group (Figure 5.).

![Figure 5. Cumulative behavioural score (0-5) following pre-treatment in seizure rats.](image)

Treatments groups were control (■), saline (■), atenolol 5 mg/kg sc. (■) and diltiazem 2.5 mg/kg sc. (■). Animals were pre-treated for 3 days, and the final dose administered 1 hr prior to KA administration. Behavioural score was taken 30 min pre-KA administration (baseline) and 180 min, 24 hr and 48 hr post-KA administration. Cumulative behavioural score was the sum of the highest recorded behavioural score from each minute, over 30 min blocks. Data presented as mean ± SEM with \( n = 4 \). *\( P<0.05 \) for all treatments compared to respective time-matched non-seizure control.
Table 3. Total behavioural scores after 180 min in pre-treatment seizure rats.

<table>
<thead>
<tr>
<th></th>
<th>Control-saline</th>
<th>Saline-KA</th>
<th>Atenolol-KA</th>
<th>Diltiazem-KA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulative behaviour score</td>
<td>17 ± 7</td>
<td>488 ± 58*</td>
<td>388 ± 77*</td>
<td>386 ± 95*</td>
</tr>
<tr>
<td>Total wet dog shakes</td>
<td>3 ± 2</td>
<td>351 ± 89*</td>
<td>264 ± 83</td>
<td>273 ± 84</td>
</tr>
</tbody>
</table>

Behavioural activity over 180 min recording period following IH KA administration. Cumulative behavioural score was the sum of the highest recorded behavioural score from each minute. *P<0.05 compared to control.

3.2 ECG Data

3.2.1 Heart rate

Control animals had a mean HR of 331 ± 17 b.p.m. which did not change over the course of the study (Figure 6.). KA administration resulted in a significant 17% increase in mean HR, when compared to baseline (458 ± 22; P<0.05, Figure 6). This increase presented at 20 minutes post seizure induction and persisted for the duration of the 180 minute recording period. Analysis of recordings at the 24 and 48 hour time points showed this elevation was not sustained and HR had diminished to baseline levels (396 ± 30 b.p.m. and 365 ± 20 b.p.m.; Figure 6). Comparatively, pre-treatment with atenolol prevented this seizure-induced increase in HR, with these animals reaching a mean HR of 350 ± 19 b.p.m. during seizure (P<0.05 vs. saline-KA group). In addition, atenolol pre-treatment also significantly reduced HR at the 24 hour time point (P<0.05 vs. saline-KA Figure 6). Administration of diltiazem prior to seizure-induction also significantly reduced HR, by up to 23%, compared to saline-KA seizure rats (P<0.05, Figure 6). However, it must be noted that this attenuation of the HR response to seizure did not present in the diltiazem pre-treated animals until 80 minutes post-KA administration. HR was still significantly reduced at the 24 hour time point in the diltiazem-KA group, compared to saline-KA (325 ± 5 vs. 396 ± 30 b.p.m., P<0.05). Furthermore, statistical comparisons between these two drug treatment groups indicated that atenolol pre-treatment effectively reduced HR, by up to 28% more than the response elicited by diltiazem during the initial 80 minutes post-KA (P<0.05, Figure 6).
3.2.2 QTc interval

The control group showed no significant change in QTc interval over the course of the study (Figure 7.). KA administration resulted in a significant increase in the QTc interval, which remained prolonged throughout the entire 180 min recording period, when compared with control (0.042 ± 0.002 vs. 0.054 ± 0.003 seconds, \( P<0.05 \)). Interestingly, QTc prolongation was sustained up until the 24 hr time point, with a 21 % increase from control (\( P<0.05 \), Figure 7.). Pre-treatment with atenolol prevented this prolongation, with QTc intervals remaining at baseline levels throughout the 180 min recording period (\( P<0.05 \) vs. saline-KA, Figure 7.). Furthermore, this attenuation of QTc interval prolongation was sustained, with a significant 38% and 25% decrease at both 24 and 48 hours post-KA, respectively (\( P<0.05 \) vs. saline-KA). Diltiazem significantly reduced the QTc interval, however, this did not become effective
until 60 minutes post-KA ($P<0.05$ vs. saline-KA, Figure 7). Analysis of this data revealed that pre-treatment with diltiazem resulted in QTc intervals similar to that seen in the control group (mean QTc over 180 min, 0.054 ± 0.0027 vs. 0.046 ± 0.0038 sec; $P>0.05$). In addition, a significant 26 % decrease was recorded at the 24 hr time point, compared with saline-KA ($P<0.05$). Comparison of atenolol and diltiazem showed no significant difference between the two treatment groups in reducing QTc prolongation.

Figure 7. QTc interval (sec) following pre-treatment in seizure rats. Treatment groups were control (●), saline (■), atenolol (♦) and diltiazem (▲). Administration of KA was at t=0. QT interval was measured and adjusted for HR across 5 minute blocks, with 30-minute blocks averaged at 24 h and 48 h. Data presented as mean ± SEM with $n=4$. *$P<0.05$ control compared to saline+KA, †$P<0.05$ atenolol compared to saline+KA, ‡$P<0.05$ diltiazem compared to saline+KA.
3.2.3  *T wave amplitude*

T wave amplitude was normalised to the last 10 minutes of baseline recording to account for differences in proximity of the ECG recording electrode. Analysis of this data revealed that there was minimal statistical difference between the different treatment groups before seizure. KA administration provoked a significant increase in T wave amplitude by 70 minutes post-KA however, this increase was not sustained (*P*<0.05 vs. control, Figure 8.). Pre-treatment with atenolol had no significant effect on T wave amplitude. Diltiazem administration also had a minimal effect on T wave amplitude over the course of the study however a significant decrease was seen at 130-150 and 165 minutes post-KA (*P*<0.05 vs. saline-KA, Figure 8).

![Figure 8](image-url)

*Figure 8. T wave amplitude (normalised, % baseline) following pre-treatment in seizure rats. Treatment groups were control (●), saline (■), atenolol (◆) and diltiazem (▲). Administration of KA was at t=0. T wave amplitude was measured across 5 minute blocks, with 30-minute blocks averaged at 24 hr and 48 hr. Data presented as mean ± SEM with *n* = 4. *P*<0.05 control compared to saline-KA, *P*<0.05 diltiazem compared to saline-KA.*
4 Summary

The findings of this study clearly demonstrated the high level seizure activity and detrimental ECG changes that occur following KA-induced seizures. Administration of KA directly into the hippocampus resulted in an immediate increase in seizure behaviours across all treatment groups, with atenolol and diltiazem pre-treatment having no effect on cumulative seizure scores. These high level seizure behaviours were sustained for the duration of the 3 hour recording period, with animals often progressing to generalised seizure activity. The intense seizure activity seen in the current study was associated with the development of cardiac dysfunction. A rapid rise in HR was seen to occur following KA administration, with animals developing sustained tachycardia and prolongation of the QTc interval.

Pre-treatment of animals with the β-blocker, atenolol, or CCB, diltiazem, significantly attenuated the development of these ictal-induced ECG abnormalities. Atenolol pre-treatment was found to significantly decrease both HR and QTc interval from the saline-KA group, with this profound cardiac effect sustained throughout the study. Pre-treatment with diltiazem produced a similar decrease in HR and QTc interval however, this therapeutic effect commonly did not present until at least 1 hour post-KA. Furthermore, when compared with atenolol pre-treated animals, diltiazem-mediated therapeutic actions were sustained for a shorter period of time. The current study demonstrates the benefits of prophylactic treatments with atenolol and diltiazem in epilepsy. In addition, these findings provide a rationale for further investigation into the potential cardioprotective effects of therapeutic intervention with these drugs during seizure.
Post-treatment with Atenolol and Diltiazem
5 Methods

5.1 Reagents

Reagents used for the following experiments were the same as those used in section 2.1.

5.2 Animals

Acquisition and care of animals was carried out as described in section 2.2.

5.3 Seizure induction and drug treatment

5.3.1 Seizure induction

The same protocol, as described in section 2.3, was used for the induction of seizures in the following post-treatment experiments.

5.3.2 Post-treatment protocol

Animals were randomly assigned to one of four different groups in this post-seizure intervention study \((n = 5\) animals per group). Animals were once again treated with saline sc. (saline-KA), 5 mg/kg atenolol sc. (Atenolol-KA) or 5 mg/kg bid. diltiazem sc. (Diltiazem-KA) and compared to the non-seizure saline control group receiving an equivalent volume of saline-vehicle. Therapeutic intervention with these drugs initially occurred at 60 min post-KA and continued daily until the animals were euthanised 7 days post-seizure induction.

5.4 Blood Pressures

A non-invasive tail cuff linked to an ML750 PowerLab unit was used in the current study to measure changes in systolic BP (SBP). Three repeat BP readings were taken immediately prior to KA administration and again at the 1 hr, 3 hr, 24 hr, 48 hr and 7 day time points. These measurements were analysed using the LabChart v.6 (ADInstruments, Dunedin, New Zealand) and the average of the three SBPs used for analysis.
5.5 Arrhythmogenesis Testing

Assessment of the animal’s susceptibility to pharmacologically-induced arrhythmias was carried out upon the completion of the 7-day behavioural recording, using the pro-arrhythmogenic voltage-gated sodium channel opener, aconitine (0.5 mg/kg, sc.). Aconitine binds to voltage-gated Na⁺ channels, prolonging the open state, and leading to the development of early-after and delayed-after depolarisations within the heart (as reviewed by Catterall, 1980). The use of this compound results in all animals developing arrhythmias. Following the administration of aconitine, ECG activity was recorded continuously and the latency to arrhythmia onset was measured. Specifically, the time to the presentation of premature ventricular contraction (PVC) and ventricular tachyarrhythmia (VT) was measured. Upon the appearance of ventricular fibrillation, animals were immediately euthanised as described below.

5.6 Euthanasia and Tissue Collection

Animals were anaesthetised 48 hr or 7 days following KA or saline administration using a halothane induction chamber, with anaesthesia maintained using a halothane nose cone.

5.6.1 Heart excision

Following the abolishment of the pedal withdrawal reflex, autopsy scissors were used to perform a rapid thoracotomy. The heart was immediately excised, rinsed in ice-cold saline and perfused through the aorta in a retrograde manner with saline (0.9% NaCl, 4°C). The heart was arrested in diastole using 5 ml of KCl solution (20 mM KCl in 0.9% saline) and perfused fixed with 10% neutral buffered formalin. The hearts underwent further immersion-fixation overnight at 4°C and then stored (3 days) in 70% ethanol at 4°C until histological processing (Read et al., 2014a).

5.7 Histological and Immunohistochemical Studies

5.7.1 Cardiac Tissue Preparation and Embedding

Cardiac ventricular sections were cut transversely at 2, 4 and 6 mm from the apex and processed using the Excelsior ES tissue processor (Figure 9.) (Thermo Scientific, New Zealand). The tissue was dehydrated using sequentially increasing series of ethanol
concentrations (70-100%), cleared in xylene and block embedded in paraffin wax (60°C). Sectioning of the paraffin wax-embedded ventricles was then carried out. Pre-trimmed blocks were cooled for 10 min and cut in 4 µm thick sections. The tissue sections were smoothed out by floating on a 40°C water bath surface and mounted onto an adhesive Dako Flex IHC slide (Dako, Glostrup, Denmark). The slides are then baked for 1 h at 60°C. Processing of the tissue, wax embedding and sectioning was carried out with the help of the Histology Unit Hercus, Department of Pathology, University of Otago (Dunedin, New Zealand).

![Image](image.png)

**Figure 9. Representative image of the three levels at which the heart tissue was cut.** Animal hearts were sectioned at 2, 4 and 6 mm superior from the apex, producing an apical (A), mid-apical (B) and basal (C) ventricular tissue section. Taken with permission from Millen (2014).

### 5.8 Immunohistochemistry

#### 5.8.1 ApopTag

Detection of ApopTag® positive cells within cardiac sections was carried out using the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Billerica, USA). Sections were washed in xylene for 10 min and rehydrated through descending concentrations of ethanol (100%, 100%, 95%, 85%, 50%) for 4 min each. A further 4 min wash was carried out in phosphate buffered saline (PBS, pH 7.4) before a Dako pen (Dako, Glostrup, Denmark) was used to create a water-repelling circle around the tissue sections.

The use of formalin fixation procedures results in the formation of protein cross-links that can mask the antigenic sites of tissues and thereby cause false-negative staining (D'Amico *et al*., 2009). Sections were therefore incubated for 20 min with Proteinase K (1:1000) to unmask
epitopes before being washed in distilled water (2 x 2 min). Endogenous peroxidases were blocked by incubating sections with 3.0% hydrogen peroxide in PBS for 10 min. Sections were then washed in PBS (2 x 5 min). Excess liquid was gently tapped off the slides and sections were incubated with equilibration buffer (75 μL/5 cm²) for 15 min in a humidified chamber.

Following this incubation period the slides were once again gently tapped to remove excess liquid. Sections were then incubated with working strength terminal deoxynucleotidyl (TdT) enzyme (55 μL/5 cm²) for 60 min in a humidified chamber at 37°C. After 1 hr the TdT enzyme was gently tapped off and the sections incubated with pre-warmed proprietary kit stop-wash buffer for 30 min in a humidified chamber at 37°C. Following incubation, sections were washed in PBS (3 x 2 min) before anti-digoxigenin conjugate (65 μL/5 cm²) was applied to each section and allowed to incubate for 30 min at room temperature (RT). Tissue sections were then washed in PBS (4 x 2 min).

The use of 3,3-diaminobenzidine (DAB) causes the tissue section to undergo a chromogenic reaction and was used to visualise nuclei undergoing apoptosis. A DAB Substrate Kit (Vector Laboratories, Burlingame, USA) was used to prepare a DAB solution that consisted of stock solution of buffer (0.04 ml, pH 7.5), DAB (0.1 ml) and hydrogen peroxide (0.04 ml) in 5.0 ml of distilled water. Following the PBS wash, sections were incubated with the freshly made DAB solution, under low light conditions, for a period of 10 min and then washed in distilled water (5 min). Counterstaining of tissue sections was then carried out with Gills #2 haematoxylin (10 sec) before being rinsed under cold running tap water (5 min).

Following counterstaining, the sections were dehydrated in ascending concentrations of ethanol (50%, 85%, 95%, 100%, 100%) for 3 min each and cleared in in xylene for 30 sec. Finally, slides were cover-slipped using a mountant mixture of distyrene, a plasticizer and xylene (DPX).

5.8.2 CD68

Tissue sections were dewaxed with xylene (2 x 5 min) and rehydrated in descending ethanol washes (100% for 3 min, 95%, 85%, 50% for 1 min) to PBS (3 min). Following rehydration, tissue sections underwent antigen retrieval using sodium citrate buffer (10mM, pH 6) at 95°C for 30 min, then cooled at RT for a further 20 min. Two PBS washes were then carried out (5 min). Following exposure of tissue epitopes, plant-based animal-free blocker (AFB, Vector
Laboratories, Burlingame, USA) was used to inhibit non-specific binding of IgG. Sections were incubated in AFB (diluted 1:5 in ddH2O) for 2 hr, in a humidified chamber. This was followed with a 10 min wash in 1% bovine serum albumin (BSA)/PBS. Sections were then incubated with the primary antibody, CD68 (clone ED1; mouse anti-CD68 monoclonal IgG; Abcam, Cambridge, UK) diluted 1:100 in 1% BSA/PBS overnight, in a humidified chamber at 4°C.

Slides were washed in PBS following overnight incubation (2 x 10 min). Endogenous peroxidase activity was next quenched with 0.3% H2O2 in PBS for 10 min. Sections were then washed in PBS (3 x 5 min) before undergoing incubation with the secondary antibody (HRP-labelled goat anti-mouse polyclonal IgG, 1:500 dilution; Pierce, Rockford, USA) for a period of 90 min. Following this incubation period, sections were washed with PBS (3 x 5 min) and incubated with DAB solution (as described in section 1.3.1) for 14 min, under low light conditions counterstained with Gills #2 haematoxylin (as described in section 2.3.1). The sections were dehydrated in ascending concentrations of ethanol (50%, 85%, 95% for 1 min and 100% for 3 min) and cleared in xylene for 30 sec. Finally, tissue sections were coverslipped using DPX mountant.

5.8.3 Picrosirius Red Staining

Picrosirius red staining, a marker of collagen I and III, was carried out to quantify the levels of fibrotic deposition within cardiac tissue. Sections were dewaxed with xylene for 10 min and rehydrated through a series of alcohol washes (100% for 3 min, 95%, 85%, 50% for 1 min) to water (3 min). Sections were then incubated in 0.1% Picrosirius Red for 1 hour at RT. Following incubation, sections were rinsed in water and washed in acidified water (0.1 N) for 2 min. Tissues were once again rinsed in water before undergoing counterstaining with Fast Green for 45 seconds. This was immediately followed with rapid rehydration in series of alcohol washes (50%, 85%, 95% for 1 min and 100% for 3 min). Sections were then cleared in xylene (30 sec) and coverslipped using DPX mountant.

5.9 Immunohistochemistry Analysis

Digital images of each slide were taken at 20× magnification using the Aperio Scanscope CS2 image capture device (Aperio Technologies, Vista, USA). Digital analysis of these
images was carried out using the ImageScope (Aperio Technologies, Vista, USA) and Adobe Photoshop CS3 (Adobe Systems Incorporated, San Jose, USA) software packages.

5.9.1 Oedema

Adobe Photoshop CS3 was used to quantify the level of oedema within ApopTag stained tissues sections. A screenshot of each ventricle was taken and imported into Photoshop. The outside and inner ventricle of each heart was traced around before the image background was removed, leaving only the tissue section. The percentage of oedema was calculated as the number of background pixels divided by the total number of pixels in that section.

5.9.2 ApopTag

The total number of ApopTag® positive cells within each tissue section was calculated using the Nuclear Count v.9 algorithm from the Aperio ImageScope software package. This algorithm allowed the detection and quantification of the ApopTag-positive DAB-stained (brown) nuclei across the entire ventriculare section. The total area analysed was also automatically quantified, and the number of ApopTag positive cells expressed as the number of apoptotic cells per mm².

5.9.3 CD68

The total number of CD68 positive macrophage cells within each tissue section was quantified using the Nuclear Count v.9 algorithm within the Aperio ImageScope software package. The total area analysed was automatically quantified, and the number of macrophages expressed as the number of CD68 positive cells per mm².

5.9.4 Picrosirius Red

The amount of collagen within each tissue section was quantified using the Positive Pixel Count v.9 algorithm from the Aperio ImageScope software package. This software package measured the number of red pixels across each ventricular section. The total number of pixels across the entire tissue was also quantified, and the percentage of fibrosis calculated.
5.10 Statistics

All statistical analysis of data was carried out using Prism v.5 (GraphPad, San Diego, USA). Behavioural data was analysed using a non-parametric Kruskal-Wallis test. Differences in ECG parameters and BP between various treatment groups across time were determined through the use of a two-way repeated measures analysis of variance (ANOVA). Differences in oedema, immunohistochemical data and arrhythmia susceptibility between groups were analysed using a one-way ANOVA. A Bonferroni post-hoc analysis was used to calculate whether these differences between groups were statistically significant. Statistical significance was determined as $P < 0.05$. Data was presented as the mean ± standard error of the mean (SEM).
# Results

## 6.1 Behavioural Data

Analysis of post-treatment control rats showed similar findings to those previously seen in the pre-treatment study, with a cumulative behavioural score of $42 \pm 14$ across the 180 minute recording period (Table 3.). KA administration resulted in an immediate increase in total seizure score, with a 7-fold increase seen within the first 30 minutes, when compared with control ($71 \pm 10$ vs. $10 \pm 3$, respectively, Figure 10.). This increase in behavioural score was accompanied by a significant increase in the total number of level 4/5 seizure behaviours and WDS, with saline-KA animals displaying a total of $333 \pm 44$ WDS across the 180 min period (Table 3.). Atenolol- and diltiazem-treated rats had comparable seizure levels to the saline-KA group, prior to therapeutic intervention at 60 min. Examination of behavioural data from atenolol treated rats showed no differences in behavioural parameters compared to saline-KA-treated animals. However, a significant reduction in cumulative seizure score during the 150-180 min time period was observed, when compared with saline+KA animals ($32 \pm 9$ vs. $64 \pm 6$; Figure 10.). Analysis of the diltiazem group revealed that therapeutic intervention with this drug at 60 min post-KA had no effect on cumulative seizure scores or total level 4/5 behaviours at any time points recorded (Table 3.). Total WDS were not significantly increased above control levels ($160 \pm 31$ vs. $10 \pm 5$).

### Table 4. Total behavioural score after 180 minutes in post-treatment seizure rats.

<table>
<thead>
<tr>
<th></th>
<th>Control-Saline</th>
<th>Saline-KA</th>
<th>Atenolol-KA</th>
<th>Diltiazem-KA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulative behavioural score</td>
<td>$42 \pm 14$</td>
<td>$469 \pm 22^*$</td>
<td>$407 \pm 29^*$</td>
<td>$458 \pm 22^*$</td>
</tr>
<tr>
<td>Total wet dog shakes</td>
<td>$10 \pm 5$</td>
<td>$333 \pm 44^*$</td>
<td>$318 \pm 46^*$</td>
<td>$160 \pm 31$</td>
</tr>
<tr>
<td>Total level 4/5 behaviours</td>
<td>$0$</td>
<td>$29 \pm 6^*$</td>
<td>$33 \pm 14^*$</td>
<td>$36 \pm 5^*$</td>
</tr>
</tbody>
</table>

Behavioural activity over 180 min recording period following IH KA administration. Cumulative behavioural score was the sum of the highest recorded behavioural score from each minute. $^*P<0.05$ compared to control.
Figure 10. Cumulative behavioural score (0-5) following therapeutic intervention in seizure rats. Treatment groups were control (□), saline (■), atenolol (■) and diltiazem (■). Behavioural score was taken 30 min pre-KA administration and 180 min, 24 hr, 48 hr and 7 days post-KA administration. Data presented as mean ± SEM with $n=5$. *P<0.05 all treatments compared to control, ‡P<0.05 compared to saline+KA.

6.2 ECG Data

6.2.1 Heart rate

Control rats showed no significant change in HR (mean HR of $336 \pm 19$ b.p.m.) throughout the 180 min recording period (Figure 11.). Administration of KA produced a statistically significant increase in HR, which remained elevated for the remainder of the 180 min recording period (mean HR over 180 min $457 \pm 22$ b.p.m., $P<0.05$, Figure 11.). Therapeutic intervention with atenolol rapidly attenuated this increase in HR by 20% within 5 min of administration ($364 \pm 11$ vs. $457 \pm 22$ b.p.m., $P<0.05$). Furthermore, this significant decrease in HR was sustained up until the 24 hr time point ($403 \pm 27$ vs. $307 \pm 11$ b.p.m., $P<0.05$). Treatment with diltiazem was found to have minimal effect on HR during the 3 hr recording period. However, diltiazem post-treatment was found to cause a significant 22 % decrease in
HR at the 24 hr time point ($P<0.05$ vs. saline-KA, Figure 11). Atenolol administration was found to have significantly decreased HR by 20% more than diltiazem between the 65 and 140 minute time points ($364 \pm 11$ vs. $439 \pm 21$, $P<0.05$).

Figure 11. Heart rate (b.p.m.) following therapeutic intervention in seizure rats. Treatment groups were control (●), saline (■), atenolol (◆) and diltiazem (▲). Administration of KA was at $t=0$ and treatment at $t=60$. HR measured across 5 minute blocks, with 30-minute blocks averaged at 24 h, 48 h and 7 days. Data is presented as mean ± SEM with $n=5$. ‡‡$P<0.01$ all treatments compared to control, **$P<0.01$ control compared to saline+KA, ††$P<0.01$ atenolol compared to saline+KA, §$P<0.05$ diltiazem compared to saline+KA, †$P<0.05$ atenolol compared to diltiazem.

6.3 QTc Interval

QTc interval in the control group showed no significant increase from baseline, with a slight elevation only seen at the time of handling (Figure 12.). KA administration within the first 60 min resulted in QTc prolongation in all three of the treatment groups (Figure 12.). The QTc interval remained significantly prolonged in the saline-KA group up until the 24 hr time point.
Therapeutic intervention with atenolol immediately reduced the QTc interval back to control values within 6 min of administration ($P<0.05$, Figure 12.). Furthermore, atenolol treatment was also found to significantly reduce the QTc interval at both the 24 hour and 7 day time points (0.046 ± 0.004 vs. 0.062 ± 0.007 sec, 0.037 ± 0.003 vs. 0.052 ± 0.002 sec, $P<0.05$ vs. saline-KA). In comparison, post-treatment with diltiazem had no immediate effect on QTc interval, with a significant increase from the control group sustained for the first 60 minutes following administration ($P<0.05$, Figure 12.). A gradual decrease in the QTc interval could be seen to occur from the 120 minute time point onwards, with a significant decrease from saline-KA presenting within the last 30 minutes of the recording period ($P<0.05$, Figure 12.). This decrease was sustained, with a significant 29% and 27% reduction in QTc interval seen at both 24 hours and 7 days post-KA, respectively.

**Figure 12.** QTc interval (sec) following therapeutic intervention in seizure rats. Treatment groups were control (●), saline (■), atenolol (♦) and diltiazem (▲). Administration of KA was at $t=0$ and treatment at $t=60$. QT interval was measured and adjusted for HR across 1-minute blocks, every second minute, with 30-minute blocks averaged at 24 h and 48 h. Data presented as mean ± SEM with $n = 5$. *$P<0.05$ control compared to saline+KA, †$P<0.05$ atenolol compared to saline+KA, ‡$P<0.05$ diltiazem compared to saline+KA.
6.4 T wave amplitude

To account for variation in the placement of the ECG recording electrode, T wave amplitudes were normalised to baseline recordings. KA administration was found to cause a statistically significant increase at a range of time points however, this elevation was rarely sustained ($P<0.05$, Figure 13.). Therapeutic intervention with atenolol had no effect on T wave amplitude. Similarly, post-treatment with diltiazem had minimal effects, with a significant decrease in T wave amplitude only seen at 150 and 165 minutes post-KA ($P<0.05$ vs. saline-KA, Figure 13.).

![Figure 13](image.png)

Figure 13. T wave amplitude (normalised, % baseline) following therapeutic intervention in seizure rats. Treatment groups were control (●), saline (■), atenolol (◆) and diltiazem (▲). Administration of KA was at $t=0$ and treatment at $t=60$. Administration of KA was at $t=0$ and treatment at $t=60$. T wave amplitude was measured across 5 minute blocks, with 30-minute blocks averaged at 24 h and 48 h. Data presented as mean ± SEM with $n=5$. *$P<0.05$ control compared to saline+KA, †$P<0.05$ atenolol compared to saline+KA, ‡$P<0.05$ diltiazem compared to saline+KA.
6.5 **Systolic Blood Pressure**

No statistically significant differences were seen in baseline systolic BP (SBP) readings between the different treatment groups (Figure 14.). KA administration in the saline (115 ± 2 mmHg, \(P<0.01\)), atenolol (116 ± 1 mmHg, \(P<0.01\)) and diltiazem (124 ± 4 mmHg, \(P<0.01\)) treatment groups significantly increased in SBP at the 1 hour time point post-KA, when compared with control (90 ± 3 mmHg). Therapeutic intervention with saline at 60 minutes post-KA had no effect one SBP, which remained elevated above the control group at 3, 24 and 48 hours post-seizure (\(P<0.05\)). In comparison, administration of atenolol brought SBP back to control levels from the 3 hour time point until the end of the study, with a significant 25% decrease from saline-KA at 48 hours (99 ± 1 vs. 124 ± 2 mmHg, \(P<0.05\)). Similarly, post-treatment with diltiazem returned SBP readings to baseline levels at 3, 24, 48 hours and 7 days (Figure 14.). Analysis of these time points further showed that administration of diltiazem resulted in a statistically significant reduction in SBP at 24 (94 ± 4 mmHg, \(P<0.05\)), 48 hours (94 ± 6 mmHg, \(P<0.05\)) and 7 days (93 ± 2 mmHg, \(P<0.05\)), when compared with saline+KA (113 ± 2, 124 ± 2 and 111 ± 4 mmHg).

**Figure 14. Systolic blood pressure (mmHg) following therapeutic intervention in seizure rats.** Treatment groups were control (○), saline (■), atenolol (◆) and diltiazem (▲). Administration of KA was at \(t=0\) and treatment at \(t=60\). Measurements were taken 7 days after KA (ih.) administration using a non-invasive BP cuff. Data presented as mean ± SEM with \(n=5\). ††\(P<0.01\) all treatments compared to control, *\(P<0.05\) control compared to saline+KA, †\(P<0.05\) atenolol compared to saline+KA, ‡\(P<0.05\) diltiazem compared to saline+KA.
6.6 *Arrhythmia Susceptibility*

Aconitine administration at 7 days post-KA produced arrhythmias in all animals. Control animals had the longest latency to presentation of both PVC (32 ± 4 min, \(P<0.05\)) and VT (45 ± 5 minutes, \(P<0.05\)) (Figure 15.). In comparison, the saline+KA group showed a significant 2-fold decrease in the time to PVC onset, when compared with control (15 ± 4 vs. 32 ± 4 minutes, \(P<0.05\)). Saline-KA animals were also associated with a significant reduction in the latency to VT (31 ± 2 minutes, \(P<0.05\)). Therapeutic interventions with atenolol and diltiazem did not significantly reduce arrhythmia latencies compared to control however, there was no significant increase in these treatment groups when compared to saline-KA animals either (Figure 15.).

![Figure 15. Latency (min) to onset of aconitine-induced arrhythmias in treated seizure rats.](image)

Treatment groups were control (□), saline (■), atenolol (□) and diltiazem (■). Graph shows the time from aconitine administration to the initial presentation of PVC (A.) and VT (B.). Data presented as mean ± SEM with \(n = 5\). \(^*P<0.05\) compared to control.
6.7 Cardiac Oedema

Quantification of cardiac oedema as represented by interstitial cardiac tissue space at 7 days post-seizure, showed a statistically significant increase in the percentage of oedema in the hearts from saline treatment animals ($P<0.01$ vs. control; Figure 16.). The presence of cardiac oedema was seen across all three sections of the ventricle, with the majority of this structural damage localised to the subendocardium region of each section. Therapeutic intervention with atenolol significantly decreased the percentage of oedema at each level, with a 47% reduction seen at the apex alone (21 ± 3 vs. 40 ± 2 %, $P<0.05$). Similarly, post-treatment with diltiazem significantly decreased oedema to control values (Figure 16.).

6.8 Apoptotic cell Death

Immunohistochemical staining of tissue sections showed the diffuse presence of ApopTag positive cells throughout the ventricular tissue sections of all treatment groups. Analysis of the control group revealed a baseline level of ApopTag positive cells, with the apical section containing an average of 0.57 ± 0.14 ApopTag-cells/mm² (Figure 17. E.). KA administration significantly increased the number of ApopTag positive cells across all three sections, compared with control (Figure 17. E.-G.). Specifically, a 6-fold increase in ApopTag positive cells was seen within the basal-section (Figure 17. G.) of saline-KA rat hearts, when compared with control (2.24 ± 0.39 vs. 0.37 ± 0.10 ApopTag-cells/mm², $P<0.001$). Atenolol treatment significantly reduced the total number of apoptotic cells across all three sections ($P<0.05$ vs. saline-KA, Figure 17. E.-G.). Therapeutic intervention with diltiazem significantly decreased the apoptotic cell count within the basal and mid-apical sections by 54% ($P<0.01$) and 59% ($P<0.05$), when compared with saline+KA (Figure 17. F.+G.).
Figure 16. Cardiac oedema (%) following therapeutic intervention in seizure rats. Treatment groups were control (■), saline ( ), atenolol ( ) and diltiazem ( ). Oedema was measured 7 days after KA (ih.) administration. Micrographs depict representative images of cardiac oedema in control (A), saline-KA (B), atenolol-KA (C), diltiazem-KA (D) treated animals. Scale bar represents 100 µ. Oedema was quantified at the level of the apex (E), 2 mm superior (F) and 4 mm superior (G). Data presented as mean ± SEM with n = 5. **P<0.01 compared to control, †P<0.05 compared to saline+KA, ‡P<0.05 compared to saline+KA.
Figure 17. Number of ApopTag positive cells (ApopTag-cells/mm²) following therapeutic intervention in seizure rats. Treatment groups were control (☐), saline (■), atenolol (□) and diltiazem (■). ApopTag-positive cells were measured 7 days after KA (ih.) administration. Micrographs depict representative images of cardiac oedema in control (A), saline-KA (B), atenolol-KA (C), diltiazem-KA (D) treated animals. Scale bar represents 100 µ. Arrows indicate ApopTag-positive cells. ApopTag was quantified at the level of the apex (E), 2 mm superior (F) and 4 mm superior (G). Data presented as mean ± SEM with n = 5. ***P<0.001 compared to control, **P<0.01 compared to control, *P<0.05 compared to control, ###P<0.001 compared to saline+KA, ####P<0.01 compared to saline+KA, #P<0.05 compared to saline+KA, ##P<0.01 compared to saline+KA.
6.9 Inflammatory cell infiltration

Quantification of the total number of CD68 positive cells was carried out at 7 days post-KA to measure the levels of macrophage infiltration within apical, mid-apical and basal ventricular tissue sections. Control animals, showed similar levels of macrophage cells at all three levels of the ventricle (mean of 14.5 ± 2.4 CD68 cells/mm², Figure 18.). KA administration was found to increase the total number of CD68 positive cells across all three sections (*P*<0.01 vs. control, Figure 18.). Post-treatment with atenolol reduced CD68 positive cells by 122%, 50%, and 42% at the apical, mid-apical and basal level, respectively (*P*<0.05 vs. saline-KA, Figure 18. E-G). Similarly, therapeutic intervention with diltiazem caused a statistically significant decrease in the total number of macrophages (*P*<0.05, Figure 18. F + G) Diltiazem post-treatment resulted in a reduction at the basal and mid-apical sections of the heart, with a significant 39% and 42% decrease seen, respectively (*P*<0.05 vs. saline-KA, Figure 18. F. + G.).

6.10 Fibrosis

KA administration was found to cause a statistically significant increase in the level of fibrosis across all three tissue sections, when compared with control (*P*<0.05, Figure 19.). Comparatively, therapeutic intervention with atenolol caused a significant decrease in fibrosis at the basal and mid-apical levels by 50% and 39%, respectively, (*P*<0.05 vs. saline-KA, Figure 19. F.+G.). Post-treatment with diltiazem resulted in a significant 32% reduction in fibrosis at the basal level, compared to saline-KA (3.4 ± 0.3 vs. 4.5 ± 0.4 %, Figure 19. G.).
Figure 18. Number of macrophage (CD68) positive cells (cells/mm²) following therapeutic intervention in seizure rats. Treatment groups were control (☐), saline (●), atenolol (■) and diltiazem (■). Macrophages were measured 7 days after KA (ih.) administration. Micrographs depict representative images of cardiac oedema in control (A), saline-KA (B), atenolol-KA (C), diltiazem-KA (D) treated animals. Scale bar represents 100 µ. Macrophages were quantified at the level of the apex (E), 2 mm superior (F) and 4 mm superior (G). Data presented as mean ± SEM with n = 5. **P<0.01 compared to control, *P<0.05 compared to saline+KA, #P<0.05 compared to saline+KA.
Figure 19. Cardiac fibrosis (%) following therapeutic intervention in seizure rats. Treatment groups were control (☐), saline (◼), atenolol (□) and diltiazem (■). Fibrosis was measured 7 days after KA (ih.) administration. Micrographs depict representative images of cardiac oedema in control (A), saline-KA (B), atenolol-KA (C), diltiazem-KA (D) treated animals. Scale bar represents 100 µ. Fibrosis was quantified at the level of the apex (E), 2 mm superior (F) and 4 mm superior (G). Data presented as mean ± SEM with n = 5. *P<0.05 compared to control, †P<0.05 compared to saline+KA, ‡P<0.05 compared to saline+KA.
7 Summary

The findings of the current study once again clearly highlight the deleterious effects that seizures have on the heart. The administration of KA resulted in high level seizure behaviours, with a significant increase in seizure score seen across all treatment groups for the duration of the 3 hour recording period. Interestingly, therapeutic intervention with atenolol at 60 minutes post-KA resulted in a significant reduction in cumulative seizure score within the last 30 minutes of the recording. This decrease in seizure activity following atenolol administration has previously been seen in our lab and elucidates to possible cardiac- or neurological-mediated anti-convulsive activity of the drug.

Induction of seizures produced extensive structural and functional damage in the hearts of these animals. Seizures resulted in a rapid rise in HR, which remained significantly elevated until the 24 hour time point. This sustained increase was accompanied by a significant prolongation of the QTc interval, up to 7 days post-KA, and intermittent periods of T wave elevation. Furthermore, a significant rise in SBP was seen following seizure induction, with this increase still present at the end of the study in the saline-KA group. The development of these pathological alterations in cardiovascular function resulted in the establishment of profound cardiac structural damage by the 7 day time point. Saline-KA animals showed significantly higher levels of fibrosis, oedema, apoptotic cell death and inflammatory cell infiltration, which may have contributed to the faster onset and progression of arrhythmias seen in this group.

Therapeutic intervention with atenolol or diltiazem at 60 minutes post-KA was found to provide effective protection from these seizure-induced cardiac irregularities. Atenolol post-treatment immediately reduced HR and QTc interval, as well as decreasing SBP at the 48 hour time point. A decrease in seizure-induced cardiac structural damage was seen at 7 days post-KA, with these animals also showing no significant decrease in the latency to arrhythmia onset. Diltiazem post-treatment resulted in a decrease in both HR and QTc interval however, this effect was associated with a longer onset to therapeutic action when compared with atenolol rats. A sustained decrease in SBP was seen following treatment, with this effect still present at the 7 day time point. In addition to these changes in cardiovascular function, therapeutic intervention with diltiazem also significantly attenuated seizure-induced structural damage to the heart, such as fibrosis and oedema. In accordance with these findings, diltiazem treated rats showed no significant decrease in the latency to arrhythmia onset and progression.
8 Discussion

This thesis clearly demonstrates that severe cardiac damage can occur as a result of sustained seizure activity. The high level seizure activity resulting from ih. KA administration in the rat produced both structural and functional damage to the heart. Development of this cardiac injury is most likely to occur as a consequence of the sustained tachycardia and increased SBP sustained. This high cardiac workload places excess strain on the heart and lead to the establishment of fibrosis, oedema, cell death and inflammatory cell infiltration. All of these pathological factors disrupt normal myocardial activity and increase susceptibility to arrhythmias, as confirmed by the decreased latency to aconitine-induced arrhythmias seen in the saline treated seizure rats.

Atenolol and diltiazem pre-treatment effectively attenuated these seizure-induced cardiac irregularities. Administration of these pharmacological agents as a prophylactic measure prior to seizure induction resulted in a sustained attenuation in both HR and QTc interval for the 24 hour period following seizure induction. This cardioprotective effect was also found following therapeutic intervention with atenolol and diltiazem at 60 minutes post-KA. Seizure-induced elevations in HR, SBP and QTc interval, were attenuated with a reduction in the level of cardiac structural damage seen.

8.1 KA-induced seizures

Intrahippocampal KA administration in the current study produced high-level seizure behaviours, including myoclonal jerks, WDS, foaming and eventually clonic-tonic convulsions. Periods of absence-like seizure behaviours were also seen, with animals displaying abnormal resting positions, frozen posture and atypical eye blinking. This model was found to produce consistent and reproducible seizures, with all animals reaching at least level 4 behaviours (e.g. loss of balance, foaming, whole body twitches) and frequently progressing to clonic-tonic convulsions. Presentation of these high seizure behaviours occurred almost immediately after KA administration and was sustained for up to 6-8 hours, with intermittent low-level seizure activity still seen in some rats over the ensuing days (Read, 2014). These findings are similar to those reported in prior studies using the same chemoconvulsant and route of administration. Babb et al. (1995) demonstrated that infusion of KA (0.4 µg/0.2 µl) directly into the hippocampus of rats produced acute seizure
behaviours, including mastication, WDS, circling and falling. In addition, Raedt et al. (2009) also found that ih. KA (0.4 µg/0.2 µl) administration resulted in the development of discrete motor seizures, which progressed into rapidly recurring generalised tonic-clonic convulsion. Furthermore, analysis carried out in our lab using this seizure model, found that ih. KA (2 nmol/1 µl) resulted in a significant increase in EEG activity across all frequency bands (Read, 2014).

The development of these high level seizure behaviours following KA administration is the result of neuronal hyperexcitability in the brain (reviewed by Sperk, 1994). As hippocampal CA1 and CA3 regions express high levels of AMPA and kainate receptors direct administration of KA into the hippocampus results in the immediate receptor binding and activation (French et al., 1982; Monaghan et al., 1982b; Rainbow et al., 1984; reviewed by Sperk, 1994; Gluck et al., 2000; Chen et al., 2002; Lee et al., 2008; Read, 2014). This intense stimulation causes excessive depolarisation of the neuron and repetitive widespread neuronal discharge that can extend to neighbouring neurons (French et al., 1982; reviewed by Sperk, 1994; Gluck et al., 2000; Chen et al., 2002). It is this strong neuronal excitation within the hippocampus and surrounding brain regions that results in the seizure behaviours seen in the current study.

Pre-treatment with atenolol and diltiazem in this study had no significant effect on seizure behaviours, with treated animals reaching comparable cumulative behavioural scores as the saline treated seizure animals. Similarly, a significant increase in cumulative behaviour score, across all groups, was seen in the post-treatment study. Interestingly, therapeutic intervention with atenolol at 60 minutes post-KA resulted in a significant decrease in cumulative behaviour score within the last 30 minutes of the 3 hour recording period. These findings are in line with a study by Read et al., (2014b), which showed atenolol (5 mg/kg) administration in KA-induced (10 mg/kg, sc.) seizure rats resulted in a significant reduction in both seizure behaviours and high amplitude EEG spiking. This decrease in seizure behaviours following β-blocker administration is not yet fully understood but may have occurred as a result of improved cerebral perfusion following cardiac protection or through an atenolol-mediated anti-convulsive action within the brain. In line with the concept of a protected cerebral perfusion mechanism, development of challenging cardiorespiratory conditions have been shown to decrease cerebrovascular responsiveness, resulting in hypoxemia and hypoperfusion of the brain (Kreisman et al., 1991; Kobari et al., 1992; Bateman et al., 2008). Due to the demand for oxygen and glucose being higher during seizure, this decrease in blood supply to
the brain can promote neuronal ischaemic damage and a build up of glutamate, thereby progressing seizure activity (Rothman et al., 1986; Kreisman et al., 1991; Delanty et al., 1998; Bateman et al., 2008). It is therefore possible that attenuation of seizure-induced tachycardia and maintenance of normal BP levels following atenolol treatment improved cerebral perfusion, thereby reducing neuronal excitation and decreasing seizure behaviours. Alternatively, atenolol may be acting directly within the brain and inhibiting seizure progression through a neuronal-mediated mechanism. Atenolol is highly hydrophilic and under normal physiological conditions is unable to cross the blood brain barrier (BBB), however, seizure has been associated with disruption of the BBB and therefore may allow the entry of atenolol into the brain (Petito et al., 1977; Nitsch et al., 1983; reviewed by Oby et al., 2006). Recent preliminary studies in our lab using radiolabelled $^{[14]}$C have subsequently shown evidence of BBB disruption 3 hours after ih. KA-induced seizures. These findings support the hypothesis that atenolol may cross the BBB during seizure. β-adrenergic receptors are highly expressed on pyramidal cells in both the CA1 and CA3 region of the hippocampus, with activation seen to produce neuronal excitatory effects (Bramham et al., 1997; Stoop et al., 2000; Hillman et al., 2005). Blockade of these receptors by atenolol may therefore reduce neuronal excitation and attenuate seizure activity, thereby resulting in the reduced seizure behaviours seen in the current study.

These findings indicate that ih. KA administration produces consistent, reproducible seizure activity and behaviours. In addition, the use of this route of administration eliminates any potential peripheral effects of the chemoconvulsant and therefore makes it an ideal model for the study of seizure-induced cardiac damage.

### 8.2 Cardiovascular Alterations

#### 8.2.1 Tachycardia

The majority of epileptic patients are indicated to experience an increase in HR during seizure, with the development of ictal tachycardia reported in 97-100% of seizures (Nei et al., 2000; Opherk et al., 2002; Zijlmans et al., 2002; Jansen et al., 2010). This tachycardic response is supported by the current study, where KA-induced seizures resulted in an immediate increase in HR. This tachycardia was sustained throughout the 3 hour recording period, with a significant elevation in HR still present at the 24 hour time point in both studies. Similar findings have been reported in other animal seizure models, with a study by
Metcalf et al. (2009b) showing a significant increase in HR within 30 minutes of lithium-pilocarpine-induced seizures in a rat. In addition, evidence of a chronic increase in HR following seizures has also been reported with Naggar et al. (2014) showing HR was significantly elevated 7-11 months after KA-induced (10 mg/kg, single bolus ip.) seizures. Seizure-induced tachycardia can cause detrimental cardiac injury and has been linked with the development of ischaemic damage, myocardial hypertrophy and cardiomyocyte death (Packer et al., 1986; Shinbane et al., 1997; Shizukuda et al., 1998; Metcalf et al., 2009b). Furthermore, the reduced cardiac output that is consequent to tachycardia can reduce tissue perfusion to key organs thereby promoting further injury to other organs (Achten et al., 2003).

It must be noted that the use of this ih. KA seizure model was not associated with the development of bradycardia, as has been seen in other animal models using systemic administration of KA (Ferrari et al., 2008; Read et al., 2014a; Read et al., 2014b). This finding highlights the potential peripheral effects of KA on structures such as the vagal nerve and provides support for use of the ih. KA protocol as a selective model for studying seizure-induced cardiac injury.

Atenolol and diltiazem treatment in the current study produced a profound negative chronotropic effect following the induction of seizures. Treatment with atenolol for three days prior to KA administration significantly attenuated the seizure-induced increase in HR observed in saline-KA animals. Similarly, therapeutic intervention with atenolol at 60 minutes post-KA resulted in an immediate decrease in HR, which was maintained at baseline levels throughout the study. These findings are supported by Read et al., (2014b), where pretreatment with atenolol (5 mg/kg) 3 days prior to seizure induction significantly attenuated the KA-induced (10 mg/kg, sc.) tachycardia. In addition, Bealer et al. (2010) showed that atenolol (1 mg/kg, iv.) administration in electrical stimulation-induced seizure rats resulted in a significant decrease in HR to levels similar to that seen in control animals.

The negative chronotropic effects produced by atenolol are mediated through selective inhibition of the β1 receptors on the heart (Figure 20.) (reviewed by Cruickshank et al., 1991; Frishman, 2008; Frishman et al., 2011). Blockade of these adrenergic receptors reduces intracellular Ca^{2+} levels, thereby depressing SA node function, slowing AV node conduction and prolonging action potential duration (reviewed by Mansoor et al., 2009). Consequently, it is through these mechanisms that atenolol is capable of attenuating ictal-induced cardiac stimulation from both circulating catecholamines and direct innervation by sympathetic
nerves. This maintenance of HR and cardiac output at physiological levels reduces the strain put on the heart whilst also maintaining tissue perfusion (Achten et al., 2003).

**Figure 20. Atenolol and diltiazem site of action.** NA denotes noradrenaline; LTCC, L-type Ca\(^{2+}\) channel; AC, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; SR, sarcoplasmic reticulum (adapted from Lohse et al. (2003))

Diltiazem pre-treatment produced a significant decrease in HR, however the onset of this therapeutic effect was slower than the response to atenolol. In comparison, post-seizure intervention with diltiazem had no immediate effects on HR, until 160 minutes post-KA. Despite this, a significant reduction in HR was found at the 24 hours time point in both the pre- and post-treatment diltiazem studies. These negative chronotropic actions of diltiazem pre-treatment during seizure (and post-treatment at 24 hours) are consistent with those seen in previous studies (Walsh et al., 1984; Schreck et al., 1997; Semsarian et al., 2002; Demircan et al., 2005). Semsarian et al. (2002) reported a significant decrease in HR following diltiazem (1.8 mg, oral daily for 7 days) treatment in mice with hypertrophic cardiomyopathy, when compared with saline treated mice. In addition, a study by Walsh et al. (1984) examined the effects of intravenous diltiazem administration followed by oral drug dosing in patients with severe congestive heart failure. The elevated HR seen in individuals with this condition was significantly reduced by 23% within 24 hours. These results in addition to those seen in the
current study show that treatment with diltiazem is capable of reducing the increase in HR seen in a number of pathologies, including seizure (Walsh et al., 1984).

The decrease in HR produced by diltiazem treatment occurs as a result of selective blockade of slow-inward LTCC on both myocardial and vascular tissues (Chaffman et al., 1985; Darbar et al., 1996; Elliott et al., 2011). Inhibition of these channels prevents the influx of extracellular Ca\(^{2+}\) that occurs during cellular depolarisation (Figure 20) (Chaffman et al., 1985). It is this reduction in intracellular Ca\(^{2+}\) levels, particularly in pacemaker cells, which is believed to be responsible for increasing the effective and functional refractory periods of the AV node, thereby producing the negative chronotropic and inotropic actions of the drug (Chaffman et al., 1985; Elliott et al., 2011).

8.2.2 QTc Prolongation

The development of sustained tachycardia seen following seizure induction in the current study was associated with a concurrent prolongation of the QTc interval. ECG recordings from saline-KA treated rats showed an early prolongation in the QTc interval, with this increase sustained up until the 24 hour time point in both the pre- and post-treatment studies. Similar findings have been seen in other animal seizure models, with a study by Little et al. (2012) also showing a significant increase in the QTc interval, 24 hours post-seizure induction. Furthermore, Metcalf (2009a) and colleagues showed sustained disruption of the QTc interval, with lithium-pilocarpine-induced seizure rats at 10-12 days following seizure. These findings are further supported by clinical research where patients with epilepsy display QTc prolongation during seizure activity (Brotherstone et al., 2010). The prolongation of the QTc interval is representative of an increase in the time of ventricular depolarisation and repolarisation (reviewed by Morita et al., 2008; Klabunde, 2011). The development of this disruption in the electrical of the heart has been associated with an increased risk of cardiac arrhythmias, such as ‘Torsade de Pointes’ and sudden death (Morita et al., 2008; Surges et al., 2010). A number of factors have been found to contribute to this lengthening of the QTc interval, including autonomic dysfunction such as that reported during seizures (Lathers et al., 1987; Druschky et al., 2001; Devinsky, 2004; Brotherstone et al., 2010). A study by Lathers et al. (1987) using cats showed that sympathetic discharge became synchronised with the electrical discharge of pentylenetetrazol-induced seizures. This action was given the term ‘the lockstep phenomenon’ and was associated with ECG abnormalities as the synchronised
sympathetic discharge altered cardiac stimulation. In addition to this effect, the development of sustained ictal-induced tachycardia, can result in cellular hypoxia and acidosis producing patches of ischaemic damage with cardiac troponin leakage and fibrotic deposition (Metcalf et al., 2009b; Brotherstone et al., 2010; Vranyac-Tramoundanas et al., 2011; Read et al., 2014a; Read et al., 2014b). Furthermore, studies have shown that high levels of circulating catecholamines, such as adrenaline, can interact directly with the myocardium, producing hypercontraction band necrosis and prolonged cellular depolarisation (Lee et al., 2003; Vranyac-Tramoundanas et al., 2011; Read et al., 2014a). In addition, the development of these ventricular microinfarcts following seizure have been reported to act as substrates for arrhythmogenesis as they are capable of disrupting the normal wave of depolarisation travelling through the myocardium and potentially leading to an increase in the QTc interval (Engelman et al., 2010). Studies have suggested that this QTc prolongation is a key pathological factor contributing to the development of arrhythmias and sudden death seen epileptic individuals (Brotherstone et al., 2010; reviewed by Surges et al., 2010).

Pre-treatment and post-treatment of animals, with either atenolol or diltiazem, attenuated the significant increase in QTc interval seen in the saline-KA group. The efficacy of these drugs at decreasing the QTc interval was similar in the pre-treatment study however post-treatment with atenolol produced a more profound and sustained therapeutic effect. This reduction in response to baseline values following drug treatment occurred at intermittent time points and was sustained up until 24 hours post-KA in both pre- and post-treatment animals. These results were supported by Little et al. (2012) studying electrically-induced SE in rats. Pre-treatment with atenolol (1 mg/kg, iv.) in this study was found to prevent prolongation of the QTc interval when assessed at 24 hours post-seizure induction (Little et al., 2012). In addition, a study by Bealer et al. (2010) found that treatment with atenolol (1 mg/kg, iv.) significantly attenuated the increase in QTc interval seen at 12-14 days in lithium-pilocarpine-seizure rats. Similarly, the use of diltiazem has also been reported to attenuate prolongation of the QTc interval (Darbar et al., 1996). A study by Darbar et al. (1996) showed that pre-treatment of patients with diltiazem (0.35 mg/kg bolus iv followed by 0.14 mg/min infusion for 30 minutes) significantly attenuated the development of adrenaline-induced QTc prolongation. In summation, these findings show that treatment with atenolol and diltiazem, in particular pre-treatment, can attenuate seizure-induced QTc prolongation.
8.2.3  T Wave Amplitude

The presentation of the T wave in the cardiac cycle is indicative of ventricular repolarisation (Matyas et al., 1976; Yan et al., 1998). Alterations in the amplitude and presentation of this wave form can be used as markers of cardiac damage and electrolyte irregularities, such as ischaemia and hyperkalemia (Yan et al., 1998; Mattu et al., 2000; Montague et al., 2008). The induction of seizures had minimal effect on T wave amplitude in both pre- and post-treated animals. A significant increase in T wave amplitude was only seen at a few intermittent time points in saline-KA treated animals, but this was not sustained. This lack of significance may be in part due to the small n number and therefore a larger sample size could provide a more accurate result. Using a more highly powered study, studies in our laboratory with larger n numbers have found a sustained significant increase in the amplitude of the T wave following ih. and systemic KA (Read et al., 2014a; Read et al., 2014b). Read et al. (2014b) showed a sustained 150% increase in T wave amplitude at 60 minutes post-KA (10 mg/kg, sc.).

Administration of atenolol was found to have no effect on T wave amplitude over the course of the study. In comparison, pre-treatment with diltiazem produced a short period in which T wave amplitude was significantly decreased from the saline-KA group, however this was not sustained. Furthermore, post-treatment with diltiazem had minimal effects on this ECG parameter. As stated earlier, further experiments to increase the sample size of the study may provide additional information and a better representation of the effect of these drugs. Despite the lack of significance, it can be seen from this data that atenolol and diltiazem treatment showed a trend towards decreasing T wave amplitude. This result is consistent with other work by Read et al., (2014b) which showed atenolol pre-treatment significantly attenuated the seizure-induced increase in T wave amplitude.

8.2.4  Blood Pressure

Previous clinical and animal studies have varyingly reported an increase, decrease or no change in BP during the ictal and inter-ictal periods (van Buren, 1958; Magnaes et al., 1974; Lou et al., 1979; Beig et al., 2009; Surges et al., 2009; Damasceno et al., 2013; Naggar et al., 2014). This varied BP response to seizures may stem from neuronal excitation of different brain regions involved in autonomic control (Kabat et al., 1935; Green et al., 2005). Despite this difference, the vast majority of studies examining this parameter have reported an increase in BP during seizures (van Buren, 1958; Lou et al., 1979; Natelson et al., 1998; Beig
et al., 2009; Surges et al., 2009; Bealer et al., 2010; Little et al., 2012; Damasceno et al., 2013). The results presented in the current study showing a sustained rise in SBP following seizure are therefore in line with these earlier published findings. SBP increased significantly by the 1 hour time point and remained elevated for up until 7 days post-KA. Similar findings were reported by Damasceno et al. (2013), where Wistar audiogenic seizure rats suffered a significant elevation in SBP (133 ± 3 mmHg) during the inter-ictal phase. A significant increase was also seen by Beig et al. (2009), with a rise in MABP observed in rats following the induction of pentylentetrazole seizures. In addition, Little (2012) and colleagues supported these findings found a significant increase in MABP was still present 24 hours post-seizure in the electrical stimulation-induced seizure rat. These findings are in line with early clinical studies in epileptic patients, which showed the development of definite hypertension (SBP >160 mmHg, diastolic BP >95 mmHg) during seizures (van Buren, 1958; Lou et al., 1979). A study by Lou et al. (1979) in newborn babies found a significant 100% increase in MABP occurred during generalised tonic-clonic convulsions. Seizure-induced hypertension is capable of producing further cardiac damage as a result of elevated afterload. The rise in arterial pressure increases cardiac workload and oxygen consumption, as the myocardium must now contract at a higher force to overcome these forces opposing ventricular ejection (Little et al., 1982; Nolan et al., 1988). In addition, the elevated BP can result in increased systolic wall stress (afterload), promoting hypertrophy and apoptotic cell death (Cheng et al., 1995). This increased stress on the heart further contributes to the damage already sustained from seizure-induced tachycardia.

Regulation of BP involves input from a number of structures in the body, such as the baroreceptors, chemoreceptors and kidneys (reviewed by Guyenet, 2006). For this reason, changes in BP can occur from alterations in HR, cardiac output, vascular resistance, activity of the adrenal medulla, Na⁺ reabsorption in the kidneys and neuronal activity in the hypothalamus/rostral ventrolateral medulla/nucleus of the solitary tract (reviewed by Guyenet, 2006). It is therefore possible that the increase in BP seen in the current study may be result of seizure-mediated stimulation of brain regions such as the hypothalamus or rostral ventrolateral medulla. Activation of these areas stimulates barosensitive sympathetic efferent fibres and therefore can elevate BP through increased HR and CO, arteriole vasoconstriction or increased fluid retention (Guyenet, 2006). In addition, the high levels of circulating catecholamines in the plasma may also promote hypertension through an interaction with α₁ adrenergic receptors on blood vessels, therefore inducing vasoconstriction (Heros et al., 1983;
Natelson et al., 1998; Sakuragi et al., 2007; Lyon et al., 2008; Thomas, 2011). Furthermore, impaired baroreflex sensitivity has been reported in epileptic individuals and therefore may be involved in the sustained increase in BP seen in the current study (Lanfranchi et al., 2002; Kaya et al., 2005; Dütsch et al., 2006). Impairment of this pathway would negatively affect the cardiac and renal physiological compensatory mechanisms controlling BP (Lanfranchi et al., 2002; Dütsch et al., 2006; Guyenet, 2006).

Therapeutic intervention with atenolol or diltiazem in the current study resulted in a significant decrease in SBP. Support of these anti-hypertensive effects has been seen in other studies examining atenolol- and diltiazem-mediated actions on BP. A study by Little et al. (2012) using electrical stimulation-induced seizure rats showed atenolol (1 mg/kg, tail vein) treatment resulted in a significant reduction in MABP. In addition, a study by (Xu et al., 2004) reported a significant decrease in SBP following atenolol (10 mg/kg) treatment in spontaneously hypertensive rats. However, it must be noted that current literature now challenges the therapeutic value of atenolol in controlling hypertension, with studies showing it may be less effective in elderly patients and is associated with higher mortality rates when compared with the use of other anti-hypertensive agents (Carlberg et al., 2004). Amodeo et al. (1986) found that diltiazem administration in hypertensive patients produced a significant decrease in MABP and reduced left ventricular hypertrophy. Furthermore, Chrysant et al. (1994) showed that treatment with diltiazem (90 mg, bid.) significantly reduced both SBP and BP load in individuals with moderate essential hypertension.

These BP lowering properties of both diltiazem and atenolol allow for sustained regulation of normotensive BP levels and therefore provide justification for the decrease in SBP seen in the current study. Furthermore, these findings highlight the beneficial cardioprotective effects that can be attained from atenolol- and diltiazem-mediated BP regulation. CCBs, particularly the dihydropyridine class, as well as thiazide diuretics and RAS modulators have now largely replaced the use of β-blockers in hypertension.

8.3 Cardiac Structural Alterations

The cardiac consequences of seizures commonly involve both conduction abnormalities, as previously discussed and structural damage to the heart. The induction of seizures in the current study resulted in a number of pathological changes to the structure of the myocardium, including fibrotic deposition, oedema, cell death and inflammatory cell
infiltration. As these findings are consistent with the cardiac pathology described in epileptic patients at post mortem the effects of therapeutic intervention on these pathological features were also examined (Natelson et al., 1998).

8.3.1 Interstitial Oedema

The accumulation of fluid within the interstitial spaces of the heart has detrimental effects on cardiac function (Dongaonkar et al., 2010). A number of factors have been linked to the development of interstitial oedema, including acute myocardial ischaemia and arterial hypertension; both of which are associated with seizure (Laine et al., 1991; Nilsson et al., 2001; Friedrich et al., 2008). In addition, post-mortem examinations of SUDEP patients have shown a significant increase in heart weight as well as the presence of interstitial oedema along the myocardial conductive tissue (Kloster et al., 1999; Stöllberger et al., 2004). These findings are in line with the current study, which showed a significant increase in the level of interstitial oedema following seizures. Examination of the interstitial space at the apical, mid-apical and basal levels of the heart showed that the saline-KA animals had significantly higher levels of interstitial oedema at 7 days post-seizure induction. Similar findings have been seen in other studies, with Read et al., (2014b) and colleagues reporting KA-induced (10 mg/kg, sc.) seizure rats had a significant 31% increase in the level of ventricular oedema at 48 hours post-seizure induction.

The presence of this excess interstitial fluid increases the stiffness of the heart, therefore decreasing compliance of the left ventricle and reducing cardiac output (Pogatsa et al., 1976; Laine et al., 1991). This inability of the heart to contract and pump blood correctly is of serious concern during high stress situations, such as seizure, as this will further exacerbate the decrease in cardiac output that has already occurred from ictal-induced tachycardia (Laine et al., 1991; Walton et al., 1995; Little et al., 2012). These compounding pathological alterations can result in damage of central and peripheral organs, including the brain, whilst also worsening cardiac injury from decreased coronary blood flow (Rubboli et al., 1994; Dongaonkar et al., 2010). Over time this damage to the heart is intensified with the expanding space between myocardial cells, increasing the oxygen diffusion distance (Ziegler et al., 1971; Dongaonkar et al., 2010). In addition to these detrimental alterations, the development of myocardial oedema has also been shown to trigger fibrotic deposition in the heart and
therefore may cause further damage, increasing the risk of arrhythmia through the formation of ischaemic microinfarcts (Davis *et al.*, 2000; Metcalf *et al.*, 2009b; Read *et al.*, 2014a).

Post-treatment with either atenolol and diltiazem in the current study significantly reduced the level of cardiac oedema suggesting a cardiovascular action. Analysis of animal hearts at 7 days post-KA showed that therapeutic intervention with these drugs significantly decreased interstitial oedema at the basal, mid-apical and apical ventricular levels. Support of these therapeutic effects can be seen in other animal studies, with Read *et al.*, (2014b) reporting a significant reduction in the level of ventricular oedema following atenolol treatment in KA-induced (20 mg/kg, sc.) seizure rats. To our knowledge no other studies have examined the effect of diltiazem on myocardial oedema. The attenuation of this seizure-induced increase in interstitial fluid following drug treatment is likely due to the negative chronotropic and inotropic properties of atenolol and diltiazem. This reduction in both HR and the force of cardiac contraction may therefore allow for a significant increase in the rate of myocardial lymph flow and therefore drainage of interstitial fluid (Dongaonkar *et al.*, 2010).

These findings demonstrate that seizures result in the development of detrimental myocardial oedema and that therapeutic intervention with atenolol or diltiazem can significantly attenuate this fluid build up.

8.3.2 Fibrosis

The link between seizure-induced sympathetic discharge and myocardial fibrosis has already been well established in epilepsy, with SUDEP patients commonly showing evidence of perivascular and interstitial fibrosis (Devinsky *et al.*, 1986; Natelson *et al.*, 1998; Tigaran *et al.*, 2003; Woodruff *et al.*, 2003; Simona *et al.*, 2005; Jansen *et al.*, 2010). These findings are supported by the current study, which demonstrates an increase in fibrosis throughout the myocardium, following seizures. This increase in fibrotic deposition at 7 days post-seizure induction shows the ability of seizures to rapidly induce irreversible cardiac damage. Read *et al.* (2014) reported a significant increase in myocardial fibrosis as early as 48 hours following KA-induced (10 mg/kg, sc.) seizures. In addition, a study by our group (Vranyac-Tramoundanas *et al.*, 2011) demonstrated showed the development of multifocal ischaemic damage progressing to fibrotic scarring 14 days following seizure induction with both ih. (100 pmol) or ip. (2 mg/kg) domoic acid.
The increased fibrotic deposition following seizure is commonly seen to occur in perivascular or interstitial regions (Natelson et al., 1998). The cause of this damage can be associated with a number of factors that occur during seizure, including sustained tachycardia, vessel spasming, high arterial pressure, direct catecholamine-mediated damage and stimulation of myofibroblasts (Haft, 1974; Maseri et al., 1975; Lathers et al., 1988; Shizukuda et al., 1998; O'Callaghan et al., 2002; Olson et al., 2005). These microscopic lesions, which have also been found along the cardiac conduction tissue in SUDEP patients, can disrupt the wave of depolarisation within the heart and increase the risk of life-threatening arrhythmias (Kloster et al., 1999; Engelman et al., 2010; Jansen et al., 2010).

Atenolol and diltiazem post-treatment significantly reduced the level of cardiac fibrosis, seen at 7 days following seizure. Similar findings were seen in a study by Read et al., (2014b), where atenolol treatment produced a reduction in the level of cardiac fibrosis following KA-induced (10 mg/kg, sc.) seizures. Atenolol treatment is likely mediated through the negative chronotropic and inotropic pharmacological effects, as a decrease in mechanical stress and oxygen demand reduces remodelling of the cardiac tissue (Cruickshank et al., 1991; Takahashi et al., 1997; Frishman, 2008; Frishman et al., 2011). In comparison, the effects of diltiazem were also seen in a study by Semsarian et al. (2002), where diltiazem treatment in a mouse model of hypertrophic cardiomyopathy resulted in a 17-fold decrease in the amount of myocardial fibrosis. The effects are also likely mediated through the negative chronotropic and inotropic actions of the drug however, the ability of diltiazem to produce coronary artery dilation and increase myocardial oxygen supply may also be involved (Chaffman et al., 1985; Vrolix et al., 1991). Furthermore, the ability of both drugs to control calcium entry into cardiac cells, including myofibroblasts, will have a direct effect on fibrotic deposition (Sandmann et al., 2001). By regulating Ca^{2+} entry into cardiac fibroblasts, the synthesis and degradation of collagen within the heart can be controlled (Olsen et al., 1989; Takahashi et al., 1997; Sandmann et al., 2001).

These findings show that treatment with atenolol and diltiazem is capable of reducing the extent of seizure-induced myocardial fibrosis through a number of mechanisms.

8.3.3 Cell death

Apoptotic or programmed cell death within the heart has been observed following a number of cardiac insults, including rapid ventricular pacing, hypertensive pressure overload,
mechanical stretch and myocardial infarction (Cheng et al., 1995; Hamet et al., 1995; Sharov et al., 1996; Saraste et al., 1997; Heinke et al., 2001; Goldspink et al., 2003; Abbate et al., 2006). The induction of seizures in the current study resulted in increased levels of apoptotic cell death within the heart at 7 days post-KA. It must be noted that this increase in the level of apoptosis may not solely represent cardiomyocyte cell death, as double-labelling of cardiomyocytes was not carried out. The increased level of apoptosis observed at 7 days post-KA is in line with other animal studies, which have also demonstrated an increase in apoptotic cell death within the heart following seizure (Chen et al., 2002; Metcalf et al., 2009b). A study (Chen et al., 2002) by reported a 5-fold increase in the number of apoptotic cells within the hearts of the genetically mutated tremor rat, when compared with wild-type controls. In addition, Goldspink et al. (2004) demonstrated an increase in the number of apoptotic cardiomyocytes following isoprenaline administration, with these levels peaking at 3-6 hours. Interestingly, the presence of necrotic cell death was also seen at 18 hours, with the authors suggesting this to be an example of ‘secondary-necrosis’ (Goldspink et al., 2004). These findings demonstrate the ability for simultaneous apoptotic and necrotic cell death to occur in the heart and highlight the effect time can have on the type of cell death.

A number of factors can promote apoptosis, with heightened adrenergic drive one mechanism that can contribute to cardiomyocyte toxicity and death (Shizukuda et al., 1998; Chen et al., 2002; Goldspink et al., 2003). Excessive sympathetic stimulation to the heart, in conjunction with high levels of circulating catecholamines, have been shown to induce continuous \( \beta \)-adrenergic activation, which consequently results in cardiomyocyte apoptosis from \( \text{Ca}^{2+} \) overload (Shizukuda et al., 1998; Chen et al., 2002; Goldspink et al., 2003; Goldspink et al., 2004). The presence of these pathophysiological conditions in both animal models and patients with epilepsy, therefore provides a likely mechanism through which seizures induce apoptotic cell death within the heart. This increased level of myocyte death can have deleterious effects on both cardiac structure and function. The development of diffuse regions of cell loss from apoptosis results in slippage of the cells (mural translocation) within the heart wall and a reduction in the functional capacity of the myocardium, which ultimately leads to an increased workload and further damage (Cheng et al., 1995).

Therapeutic intervention with atenolol and diltiazem at 60 minutes post-KA resulted in a significant reduction in the number of apoptotic cells within the heart. Support of these findings were reported in a study by Sabbah et al. (2000), with metoprolol (25 mg, bid.) treatment attenuating cardiomyocyte apoptosis in a dog model of heart failure. Gao et al.
(2001) demonstrated similar anti-apoptotic effects with the CCB, benidipine (3 µg/kg, iv.), which was reported to decrease the levels of myocardial apoptosis in rabbits subject to ischaemia-reperfusion. Furthermore, the treatment of adult rat cardiomyocytes (in vitro) with diltiazem was found to attenuate noradrenaline-stimulated apoptosis (Communal et al., 1998). The reduction in apoptosis observed following treatment with these drugs is likely associated with improved calcium handling within the cells. The ability of atenolol and diltiazem to attenuate cardiomyocyte Ca\(^{2+}\) influx prevents the intracellular Ca\(^{2+}\) overload that is associated with excess circulating catecholamines and subsequent cardiomyocyte apoptosis (Chaffman et al., 1985; Nishida et al., 1999; Goldspink et al., 2003; Mansoor et al., 2009; Dhalla et al., 2010).

8.3.4 Inflammatory cell infiltration

Intense, sustained sympathetic drive to the heart, similar to that seen during seizure, results in cardiac damage that can subsequently initiate an inflammatory response (Mann, 1996; Murray et al., 2000; Frangogiannis, 2006a; Shimizu et al., 2007; Morel et al., 2009). It is commonly seen in other cardiac pathologies that activation of this inflammatory cascade involves cytokine-mediated recruitment of macrophages (Greenhoot et al., 1969; Frangogiannis, 2006a). This is in line with results of the current study, which demonstrates an increase in myocardial macrophage infiltration at 7 days post-seizure induction. Elevated levels of macrophages were seen across all three cardiac tissue sections, with the majority of these inflammatory cells found in perivascular regions of the heart. Similar findings were seen by Nef et al. (2007), with this study showing an increase in the number of macrophages within the myocardial tissue of patients with stress-induced (Tako-Tsubo) cardiomyopathy. The infiltration of these macrophages is beneficial in most cardiac pathologies as these cells are required for the removal of dead cellular debris and repair of the tissue (Frangogiannis, 2006a; Fischer et al., 2007). However, the chronic presence of these activated macrophages can over time be of detriment as they have been shown to adhere to myocytes and subsequently decrease the contractile function of the tissue (Simms et al., 1999).

Therapeutic intervention with atenolol and diltiazem resulted in a significant reduction in the number of infiltrating macrophage. Atenolol post-treatment was found to produce a more profound effect, with a decrease seen at all three levels measured, whilst diltiazem therapy reduced macrophage infiltration within the basal and mid-apical section. These findings are
seen in other studies, with Cimmino et al. (2011) showing metoprolol (7.5 mg, iv.) and carvedilol (5.25 mg, iv.) treatment in a pig model of acute myocardial infarction produced a significant decrease in myocardial macrophage infiltration. In addition, a study by Takatsu et al. (2012) demonstrated treatment with the LTCC blocker, amlodipine (3 mg/kg), reduced the number of infiltrating macrophage within the myocardium of rats with left ventricular hypertrophy. The ability of atenolol and diltiazem to reduce inflammatory cell infiltration following seizure is likely a result of decreased mechanical stress on the heart from the negative chronotropic and inotropic actions of both drugs.

8.4 Arrhythmogenic risk

Cardiac rhythm and conduction disorders are commonly found in epilepsy, with ECG abnormalities (excluding tachycardia) seen in up to 31% of seizures and 72% of patients (Nei et al., 2000; Opherk et al., 2002; Zijlmans et al., 2002; Surges et al., 2009). Studies, including the current one, have shown that ictal activity results in the development of structural (fibrosis and oedema) and functional (QTc prolongation and tachycardia) abnormalities, which are associated with an increased risk of arrhythmias (Nei et al., 2000; Opherk et al., 2002; Zijlmans et al., 2002; Metcalf et al., 2009b; Surges et al., 2009). The results of the current study showed that following aconitine administration at 7 days post-KA, seizure animals developed both PVB and VT significantly faster than controls. Similar findings have been seen in other models of seizure, with Metcalf et al. (2009b) showing lithium-pilocarpine-seizure rats also presented a decreased latency to PVB and VT following aconitine administration. These findings support the concept that structural myocardial injury increases susceptibility to cardiac arrhythmias.

Atenolol and diltiazem post-treatment attenuated the significant decrease in latency to arrhythmias seen in saline treated animals. Therapeutic intervention with these drugs resulted in no significant decrease from control in the time to both PVB and VT. These findings are consistent with other studies which showed that atenolol treatment in rats prevented increased susceptibility to experimentally-induced PVC and VT, at 12-14 days following seizure (Bealer et al., 2010). This anti-arrhythmic effect has also been seen following diltiazem treatment, with one study showing iv. infusion of diltiazem (0.1 mg/kg) over 2 minutes significantly suppressed aconitine-induced atrial tachycardia (Yamamoto et al., 1993). In addition, a study by Clusin et al. (1984) found diltiazem (0.5 mg/kg iv. infusion)
administration significantly increased the latency to ventricular fibrillation, in a dog model of ischaemia-induced conduction impairment.

The ability of atenolol and diltiazem to attenuate the faster arrhythmia onset seen in the saline-KA treatment group is likely due to the reduced damage seen at the time of seizure and the pharmacological action of these drugs following aconitine administration. Both of these drugs inhibited a number of the seizure-induced pro-arrhythogenic factors, including QTc prolongation, tachycardia, fibrotic deposition, myocardial oedema and elevated SBP. The decreased levels of these pathological factors reduced the extent of cardiac damage present at the time of aconitine administration. Furthermore, the control of both rate and conduction by atenolol (Class II anti-arrhythmic agent) and diltiazem (Class III anti-arrhythmic agent) when administered at the 7 day time point would have contributed to attenuating the pro-arrhythogenic effects of aconitine. The findings obtained from this experiment highlight the benefits of cardioprotective drug therapy with atenolol and diltiazem at the time of seizure and the anti-arrhythmic effects of the drugs themselves.

8.5 Comparison

The current study clearly demonstrated the efficacious cardioprotective actions of atenolol and diltiazem treatment in a rat model of seizure-induced cardiomyopathy. The attenuation of both structural and function irregularities following treatment with these drugs highlights the therapeutic benefit that can be attained with both prophylactic treatment and therapeutic intervention. The pharmacological actions of both atenolol and diltiazem involve preventing an increase in intracellular Ca\(^{2+}\) levels during cellular depolarisation (Chaffman et al., 1985; Frishman, 2008; Mansoor et al., 2009; Elliott et al., 2011; Frishman et al., 2011) Atenolol achieves this through blockade of the \(\beta_1\)-receptor on the sarcolemma, whilst diltiazem reduces Ca\(^{2+}\) levels by inhibiting LTCC in the T-tubules (Chaffman et al., 1985; Mansoor et al., 2009; Elliott et al., 2011; Frishman et al., 2011). The negative chronotropic and inotropic actions of these drugs are primarily mediated through this ability to modulate Ca\(^{2+}\) levels in cardiomyocytes. This regulation of Ca\(^{2+}\) concentration within the cardiomyocyte also likely contributes to the ability of these drugs to prevent QTc prolongation, as seen in the current study (Chaffman et al., 1985; Mansoor et al., 2009; Elliott et al., 2011). Furthermore, the decrease in SBP following drug treatment is likely mediated through different sites of action. Atenolol administration results in blockade of the \(\beta_1\)-receptors on both cardiomyocytes and
juxtaglomerular cells resulting in a drop in BP through maintenance of normal cardiac output and decreased fluid retention (Amer, 1977; Brewster et al., 2004; Mason et al., 2009). In comparison, diltiazem inhibits LTCC on vascular smooth muscle, thereby causing vasodilation of the vessel and a subsequent drop in BP (reviewed by Flynn et al., 2000; Hayashi et al., 2003; Godfraind, 2005; Elliott et al., 2011). These cardiovascular actions of atenolol and diltiazem are likely to be the primary mechanisms through which these drugs reduced seizure-induced cardiac injury in the current study.

Despite both of these drugs reducing the extent of cardiac damage, it is evident from these findings that treatment with atenolol produced a more profound and immediate therapeutic response than diltiazem. This result was of interest and may be explained by the pharmacodynamics and pharmacokinetics of these drugs. The pharmacodynamic actions of both atenolol and diltiazem involve preventing an increase in intracellular Ca\(^{2+}\) levels during cellular depolarisation (Chaffman et al., 1985; Frishman, 2008; Mansoor et al., 2009; Elliott et al., 2011; Frishman et al., 2011). Atenolol achieves this through blockade of the \(\beta_1\)-receptor on the sarcolemma, whilst diltiazem reduces Ca\(^{2+}\) levels by inhibiting LTCC in the T-tubules (Chaffman et al., 1985; Mansoor et al., 2009; Elliott et al., 2011; Frishman et al., 2011). However, despite inhibition of the LTCC, cellular depolarisation and the ensuing myocardial contraction may still occur through persistent \(\beta_1\) receptor stimulation. This sustained activation of the \(\beta_1\) receptor can cause PKA-hyperphosphorylation leading to PKA-mediated activation of the ryanodine receptor and subsequent Ca\(^{2+}\) release from the sarcoplasmic reticulum (Marx et al., 2000; Lehnart et al., 2008; Shan et al., 2010). This small rise in intracellular Ca\(^{2+}\) levels can be sufficient to stimulate calcium-induced calcium release from the sarcoplasmic reticulum thereby producing a rapid rise in Ca\(^{2+}\) levels within the cell and subsequent excitation-contraction coupling (Marx et al., 2000; Curran et al., 2007; Lehnart et al., 2008; Shan et al., 2010). This phenomenon is seen in cardiac pathologies, including heart failure, and could therefore justify the findings of the current study in which blockade of the LTCC by diltiazem was initially unable to produce a reduction in HR during seizure (Marx et al., 2000; Lehnart et al., 2008; Shan et al., 2010). In addition, this concept would support the profound negative chronotropic effects observed with atenolol treatment as this drug inhibits Ca\(^{2+}\) influx through direct blockade of the \(\beta_1\) receptor (Mansoor et al., 2009). Furthermore, this leakage of Ca\(^{2+}\) from the sarcoplasmic reticulum has been shown to promote arrhythmogenesis and sudden cardiac death and therefore may explain the increased
susceptibility of seizure-rats to aconitine-induced arrhythmias (Wehrens et al., 2003; Lehnart et al., 2008).

The pharmacokinetics of these drugs is another key factor that must be considered when evaluating the results of the current study as this may explain the difference in the time taken to achieve a therapeutic response for parameters such as HR. Atenolol is a hydrophilic drug, with a long therapeutic half life of 7-9 hours (Shanks et al., 1977; Cruickshank, 1980). This hydrophilic profile allows for rapid absorption of the drug into the bloodstream, where it will primarily stay and circulate due to the polar charge (Cruickshank, 1980). These pharmacological actions of atenolol in a physiological system therefore may explain the immediate therapeutic response seen following drug administration at 60 minutes post-seizure induction. Furthermore, the long half-life of atenolol allows for sustained therapeutic effects from once daily dosing. In comparison, diltiazem is highly lipophilic and possesses a very short half-life of 3-5 hours, with the plasma concentration of this drug directly correlating with the therapeutic actions attained (Hermann et al., 1983; Smith et al., 1983). This lipophilic profile of diltiazem suggests that sc. administration in the current study would result in the rapid expulsion of the drug from the plasma into the surrounding tissues. Furthermore, this effect is supported by studies examining the pharmacokinetics of diltiazem reporting a large volume of distribution and subsequent low bioavailability, with studies in rats reporting a bioavailability of 37% (pv. administration) (Hermann et al., 1983; Lee et al., 1991). These actions would therefore result in the slow release of the drug into the bloodstream over time and therefore may account for the delayed therapeutic response. In addition, the high level of diltiazem that is protein bound when in the circulation, 52-81%, lowers the percentage of free drug available for pharmacological action (Piepho et al., 1982). This may further contribute to the delayed onset of the therapeutic effect of diltiazem has been correlated to the plasma concentration of the drug (Downing et al., 1987). These findings suggest that the varying lipophilicity, protein binding, volume of distribution and subsequent biodistribution of atenolol and diltiazem account for the varied rate of therapeutic action seen in the current study.

8.6 Conclusion

In conclusion, this study clearly shows the detrimental structural and functional cardiac damage that occurs following seizure in an ih. KA-induced rat model (Figure 21.). The
sustained tachycardia and increased SBP that occurs following seizure induction results in the development of myocardial injury and dysfunction, such as prolongation of the QTc interval, fibrotic deposition and interstitial oedema. These pathological alterations to the heart can have detrimental consequences, with seizure animals demonstrating an increased susceptibility to aconitine-induced arrhythmias. Pre- and post-treatment of animals with the $\beta_1$ receptor blocker, atenolol, prevented the development of seizure-induced tachycardia and subsequently attenuated prolongation of the QTc interval. Similarly treatment with the CCB, diltiazem, prior to seizure induction resulted in a significant reduction in HR and QTc interval however, therapeutic intervention with this drug after seizure onset had minimal effect on these parameters. Both atenolol and diltiazem were found to reduce SBP to baseline levels. Furthermore, post-treatment with both drugs attenuated seizure induced structural damage, such as fibrotic deposition and myocardial oedema, whilst also reducing the levels of apoptotic cell death and subsequent inflammatory cell infiltration. This reduction in the level of structural damage following drug treatment likely contributed to atenolol- and diltiazem-attenuation of the seizure-induced risk of arrhythmia. These findings suggest that prophylactic treatment and pharmacological intervention with atenolol or diltiazem can provide cardiac protection in seizure-induced cardiac injury and therefore should be considered as an adjunct therapy in patients with severe epilepsy.

Figure 21. Summary diagram of proposed mechanism of seizure-induced cardiac damage and benefit of therapeutic intervention.
8.7 Methodological Evaluation

8.7.1 Animal studies

The implantation of a telemetric transmitter into the abdomen resulted in some animals \(n=2\) developing complications leading to the exemption of these rats from the study.

The sc. administration of drugs did not represent the current clinically used route of administration, as atenolol and diltiazem are usually prescribed as oral medications.

Measurement of seizure activity could have been strengthened through the analysis of both behavioural data and EEG activity. While EEG activity was recorded in this study, analysis of this recording was not undertaken to determine seizure activity.

Daily recordings of behavioural and ECG activity for the length of the 7 day study may have provided more information on ongoing cardiac changes.

8.7.2 Immunohistochemistry

Although the ApopTag kit used in this study is considered sensitive assay for apoptotic cell death there is now controversy as to whether the use of the TUNEL method can result in false-positive staining of necrotic cells (Shizukuda et al., 1998). Furthermore, double-labelling of cardiomyocytes was not carried out in the current study and therefore limits the findings of the ApopTag immunohistochemistry procedure as there is the potential for non-specific staining of dying inflammatory cells.

8.8 Future Directions

- Develop a kindling model using ih. KA – this method results in the development of spontaneous recurring seizures to provide a more representative model of epilepsy.
- Increase the length of observation post-KA administration to examine the long-term effects of seizure on the heart (3-4 month study).
- Use atenolol and diltiazem in combination with current AEDs such as phenytoin and valproate to examine the effects of concomitant cardioprotection and anti-convulsant therapy.
• Comparison of the effects of KA administration into the left versus right hippocampi on haemodynamics.

• Examine $\beta_1$- and $\beta_2$-receptor expression on the heart post-seizure to investigate possible redistribution and downregulation from sustained sympathetic activation.

• Compare the effects of diltiazem with other CCBs – is a better effect achieved with the phenyalkylamines (verapamil) or dihydropyridines (nifedipine).

• Confirm whether the myocardial cell death observed in the current study is in fact apoptotic or whether it is necrotic. This could be examined through the use of Western Blotting techniques with caspase-9, Annexin V staining or flow cytometry.

• Measurement of plasma oxygen saturation levels using an arterial cannula to determine if the cardiorespiratory dysfunction is associated with seizure and can be attenuated through a cardioprotective drug strategy.

• Western blotting to measure the levels of Kv4.2 channels on the heart as the levels of these channels have been found to decrease following seizure and may contribute to the development of cardiac arrhythmia (Bealer et al., 2012).
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