Pharmacological Intervention with Atenolol and Diazepam in Seizure-Induced Cardiomyopathy

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Abstract

Seizures have become frequently associated with an increased risk of cardiomyopathy, with tachycardia and pathological structural changes commonly reported in both clinical and animal studies. This thesis examined the hypothesis that intervention with the selective β₁-adrenergic receptor antagonist atenolol, in conjunction with the antiepileptic drug diazepam, would prevent cardiac injury in a kainic acid (KA)-induced seizure model. Male Sprague-Dawley rats (320 – 350 g) were instrumented with ECG/EEG transmitters and seizures induced by intrahippocampal delivery of KA (2 nmol in 1 µL saline). Saline, diazepam (5 mg/kg initial and 1 mg/kg bid., sc.), atenolol (5 mg/kg, sc.) or a combination of diazepam + atenolol interventions were administered at 1 hour post-KA and continued daily for the remainder of the study (7 or 14 days). High-level seizure behaviours were associated with tachycardia (482.3 ± 15 b.p.m., \( P < 0.05 \) vs. baseline) present up to 3 hours post-KA, and QTc prolongation up to 7 days post-KA, in the saline-treated group (\( P < 0.05 \) vs. baseline). Both tachycardia and QTc prolongation were significantly attenuated by treatment with atenolol alone and in combination with diazepam (\( P < 0.05 \) vs. saline group). Monotherapy with diazepam intervention effectively attenuated seizure behaviours but failed to reduce heart rate, QTc prolongation or markers of cardiac injury, and unexpectedly, heart rate was increased above saline-treated animal responses at both 24 and 48 hours post-KA (\( P < 0.05 \)). Cardiac troponin I levels were elevated up to 5-fold at 24 hours in the saline and diazepam groups (\( P < 0.05 \) vs. baseline), and this injury marker was attenuated by atenolol and combination treatments. Histological examination confirmed the presence of micro-infarcts with evidence of oedema and significant collagen I deposition in the ventricular myocardium of the saline and diazepam intervention groups. Immunolabelling also showed significant increases in apoptotic-positive cell counts (15-fold increase above naïve control, \( P < 0.05 \)) and macrophage infiltration (51.1 cells/mm²; \( P < 0.05 \) vs. naïve control) in hearts from saline and diazepam-treated animals. However, α smooth muscle actin levels, indicative of myofibroblast presence, were not altered in any treatment groups (\( P > 0.05 \) vs. naïve control).

This study clearly showed that seizure induction in the rat by intrahippocampal KA delivery results in the development of ECG changes and cardiac structural injury. β₁-receptor blockade is known to exert cardioprotective effects through the attenuation of sympathetic drive on heart rate, and by preventing the stimulation of pathological β₁-mediated pathways including apoptosis, hypertrophy, and remodelling. The evidence of diazepam-induced heart rate increases presented here, tallies with previous reports of central- and peripheral-mediated cardiac effects of diazepam. These detrimental cardiac effects of diazepam were attenuated by
the use of atenolol in the combination intervention strategy suggesting that $\beta_1$ receptor-mediated pathways may be implicated in the detrimental cardiac effects of diazepam. This thesis strongly indicates that atenolol is an effective cardioprotective adjunct therapy of value in clinical seizure management.
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Abbreviations

\([Ca^{2+}]_i\) intracellular calcium ion concentration
AED antiepileptic drug
Akt protein kinase B
AMPA \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AV atioventricular
BBB blood brain barrier
BP blood pressure
\(Ca^{2+}\) calcium ion
CaMKII \(Ca^{2+}/\)calmodulin-dependent protein kinase
cAMP cyclic adenosine monophosphate
CNS central nervous system
DZP diazepam
ECG electrocardiograph
EEG electroencephalograph
GABA \(\gamma\)-aminobutyric acid
\(G_i\) inhibitory G-protein
GPCR G-protein coupled receptor
\(G_s\) stimulatory G-protein
HCN hyperpolarisation-activated cyclic nucleotide-gated channel
HF heart failure
HR heart rate
ip. intraperitoneal
iv. intravenous
\(K^+\) potassium ion
LTCC L-type calcium channel
MAPK mitogen-activated protein kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MPTP</td>
<td>mitochondrial permeability transition pore</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>Na⁺</td>
<td>sodium ion</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>KA</td>
<td>kainic acid</td>
</tr>
<tr>
<td>NTS</td>
<td>nucleus tractus solitarii</td>
</tr>
<tr>
<td>PCO₂</td>
<td>partial pressure of carbon dioxide</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-4,5-bisphosphate 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous system</td>
</tr>
<tr>
<td>PO₂</td>
<td>partial pressure of oxygen</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SA</td>
<td>sinoatrial</td>
</tr>
<tr>
<td>sc.</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SCM</td>
<td>Stress cardiomyopathy</td>
</tr>
<tr>
<td>SE</td>
<td>status epilepticus</td>
</tr>
<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
</tr>
<tr>
<td>SpO₂</td>
<td>oxygen saturation</td>
</tr>
<tr>
<td>SUDEP</td>
<td>sudden unexpected death in epilepsy</td>
</tr>
<tr>
<td>TIC</td>
<td>Tachycardia-induced cardiomyopathy</td>
</tr>
<tr>
<td>TLE</td>
<td>temporal lobe epilepsy</td>
</tr>
<tr>
<td>TSPO</td>
<td>translocator protein</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>WDS</td>
<td>wet dog shakes</td>
</tr>
<tr>
<td>αSMA</td>
<td>α smooth muscle actin</td>
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Chapter 1: Introduction
1.1 Epilepsy And Seizures

1.1.1 Epilepsy definition, epidemiology, and aetiology
Epilepsy is a common neurological disorder with a prevalence of approximately 1 – 2% in New Zealand (Epilepsy New Zealand, 2014). Epilepsy is defined by the recurrence of unprovoked seizures. Epilepsy has various aetiologies, comprising genetic, acquired (e.g. head injury), environmental (e.g. drug-induced seizures), or unknown causes (Shorvon, 2011). Seizures have been described as “a transient occurrence of signs and/or symptoms due to abnormally excessive and synchronous neuronal activity in the brain” (Fisher et al., 2014; Fisher et al., 2005). Seizures are generally classified clinically by the type of seizure behaviour presented, which reflects the brain regions affected. Seizures are divided into two main types: partial and generalised seizures. Whereas partial seizures are confined to a focal brain region, generalised seizures involve multiple regions and include tonic-clonic and absence seizure types (Berg et al., 2010).

1.1.2 Temporal lobe epilepsy
Temporal lobe epilepsy (TLE) is the most common form of partial epilepsy, and seizures arise from the temporal lobe structures, involving the hippocampus, amygdala or parahippocampal gyrus (Bertram, 2009). Hippocampal sclerosis often accompanies TLE, and is characterised by extensive cell death in the hilus, CA1 and CA3 regions of the hippocampus, leading to subsequent hippocampal atrophy and gliosis (Bertram, 2009; Buckmaster, 2004). Other limbic sites may also exhibit pathology, including the amygdala, thalamic nuclei and entorhinal cortex (Bertram, 2009). In particular, the loss of hippocampal inhibitory interneurons may render the remaining excitatory dentate granule cells hyperexcitable, leading to a decrease in seizure threshold (Buckmaster, 2004).

1.1.3 Status epilepticus
Status epilepticus (SE) is characterised by a single prolonged seizure lasting over 5 minutes, or a series of seizures over 30 minutes without recovery of full consciousness (Knake et al., 2009). Importantly, delay in controlling SE is associated with a poorer outcome (Treiman et al., 1998). It is regarded as a medical emergency due to the high mortality rate (22 – 43%) associated with this condition (DeLorenzo et al., 1996; Logroscino et al., 2002). Approximately 34 – 50% of patients with SE have been previously diagnosed with an epileptic disorder, with other causes attributed to electrolyte imbalances, drug overdose, central nervous system (CNS) infection, stroke, or traumatic brain injury (Chapman et al., 2000).
2001; Knake et al., 2001). SE must be rapidly treated, as complications include cerebral hypoxia and oedema, respiratory failure or apnoea, and metabolic disturbances (Chapman et al., 2001). In particular, cardiovascular complications may arise including myocardial infarction (MI), blood pressure (BP) alterations, arrhythmias, cardiogenic shock, and cardiac arrest (Chapman et al., 2001). Refractory SE occurs in approximately 38 – 44% of SE cases in which first- and second-line antiepileptic drugs (AED’s) are ineffective and is associated with higher morbidity and mortality (Knake et al., 2009; Lowenstein, 2006).

Figure 1.1: Coronal Nissl-stained images showing primate (Macaca mulatta; left) and rat (Rattus norvegicus; right) brains, with hippocampus inset illustrating CA1, CA3, and dentate gyrus (DG) regions. Images adapted from Mikula et al. (2007).

1.1.4 Epilepsy and seizure mortality

Seizure-related disorders are associated with poorer health outcomes and an increased risk of death. Epileptics bear an increased mortality rate 2 – 3 fold greater than the general population, with life expectancy decreased by up to 13 years (Gaitatzis & Sander, 2004; Yuen et al., 2007). Interestingly, most fatalities occur in the postictal phase 1 – 30 days following the SE episode, with a further 43% dying within 10 years (Boggs, 2004; Logroscino et al., 2002). This acute postictal mortality may arise from complications involving pulmonary aspiration, hyperthermia, rhabdomyolysis, metabolic disturbances and cardiac arrhythmias (Chapman et al., 2001). Factors contributing to the risk of mortality in the longer term include the development of chronic stress in epileptics, genetic proneness to other disorders, increased risk of injury/accidents, and autonomic imbalances (Devinsky, 2004; Tomson et al., 2004; Tu et al., 2011a; Yuen et al., 2007).

Sudden unexpected death in epilepsy (SUDEP) accounts for approximately 17% of deaths in epileptic patients (Hughes, 2009). Mechanisms contributing to SUDEP remain elusive, but
may be related to autonomic dysfunction leading to cardiac arrhythmias or central respiratory dysfunction (e.g. central apnoea) (Bateman et al., 2010; Hughes, 2009; Lathers et al., 2008; Nashef, 1997; Shorvon & Tomson, 2011; So et al., 2000; Stöllberger & Finsterer, 2004).

1.2 Neurophysiology Of Seizures

1.2.1 Physiology of a seizure
A seizure involves the abnormal, excessive, hypersynchronous firing of cerebral neurons, arising from excessive excitation or decreased inhibition (Engelborghs et al., 2000). Spread of seizurogenic activity to motor regions may result in convulsive seizures, whilst seizure activity limited to particular brain regions may induce no obvious signs of a seizure, other than autonomic dysfunction (Baumgartner et al., 2001; Engel, 2013).

1.2.2 Physiology of neurotransmitters
Glutamate and γ-aminobutyric acid (GABA) are the main neurotransmitters within the mammalian central nervous system (CNS), eliciting excitatory and inhibitor effects, respectively (Figure 1.2) (Fritschy, 2008; Kew & Kemp, 2005). Glutamate exerts its excitatory effects on metabotropic and ionotropic glutamate receptors. Ionotropic glutamate receptors are tetrameric ligand-gated ion channels, and consists of N-methyl-D-aspartate (NMDA) receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, and kainate (kainic acid; KA) receptors (reviewed by Stawski et al., 2010). Activation of these receptors leads to depolarisation of the postsynaptic membrane, by Na⁺ (and Ca²⁺ in some subtypes) through AMPA and KA receptors, and Na⁺ and Ca²⁺ influx through NMDA receptors (Kew & Kemp, 2005). These receptors are highly expressed in the hippocampus, contributing to their implication in seizure pathogenesis (Lee & Kales, 2011).

GABA mediates its inhibitory effect through interaction with inotropic GABAₐ receptors and metabotropic GABAₐ receptors (Lee & Kales, 2011). GABAₐ receptors are pentameric Cl⁻ ion channels, which induce neuronal hyperpolarisation (Lee & Kales, 2011). GABAₐ receptors are located throughout the CNS, and are also located peripherally in the adrenal gland, pancreas, kidney, vagal nodose ganglion, and sinoatrial (SA) node, with varying putative functions (Roth & Draguhn, 2012; Watanabe et al., 2002). Binding of various modulators to allosteric sites on the GABAₐ receptor modifies the inhibitory effect of GABA at the receptor. Positive allosteric modulators include barbiturates, benzodiazepines and ethanol, with benzodiazepine binding increasing the frequency of GABAₐ channel opening (Olsen & Sieghart, 2009; Wafford, 2005). The property of GABAergic inhibition has been
exploited in a number of pharmacological agents including anxiolytics, sedatives, and AED’s (Roth & Draguhn, 2012).

Figure 1.2: Chemical structures of glutamate, kainic acid and GABA. Images adapted from Dhakal et al. (2012) and Lau & Roux (2011).

1.3 Autonomic Cardiovascular Control

1.3.1 Overview of the ANS

The autonomic nervous system (ANS) comprises the peripheral sympathetic and parasympathetic nervous systems (SNS, PNS, respectively) and the central autonomic system, and is involved in a number of functions including the visceral regulation of cardiovascular, respiratory and reproductive control (Gabella, 2001). Central control of the ANS is mediated through a complex connection of cortical and brainstem regions, including the medial prefrontal cortex, amygdala, hypothalamus, insular cortex, periaqueductal grey matter and lateral parabrachial nuclei (Figure 1.3) (Green & Paterson, 2008).

Figure 1.3: Diagram showing the complex involvement of central areas in autonomic cardiovascular control. AHN: anterior hypothalamic nucleus; LHA: lateral hypothalamic nucleus; PVN: paraventricular nucleus; PBN: parabrachial nucleus; PAG: periaqueductal grey; NTS: nucleus tractus solitarii; nAmb: nucleus ambiguus; DMNV: dorsal motor nucleus of the vagus; RVLM: rostral ventrolateral medulla. Adapted from Green & Paterson (2008).
1.3.2 Parasympathetic cardiovascular control

Cardiac parasympathetic preganglionic cell bodies lie in the nucleus ambiguus and the dorsal motor nucleus of the medulla (Jansen & Lagae, 2010). These neurons project to the heart via the vagus nerve and form ganglia in the cardiac fat pads, where they subsequently innervate mainly the atria and, to a lesser extent, the ventricles (Triposkiadis et al., 2009). Postsynaptic parasympathetic terminals release mainly acetylcholine onto cardiac muscarinic receptors, primarily M₂ receptors, which are G-protein coupled receptors (GPCR’s) and are highly expressed at SA and atrioventricular (AV) nodes (Olshansky et al., 2008). The PNS can modulate cardiac function, both through central inhibition of the sympathetic system, as well as through direct cardiac M₂ receptor activation, resulting in reduced heart rate (negative chronotropy) and AV conduction (negative dromotropy) (Jansen & Lagae, 2010; Olshansky et al., 2008).

1.3.3 Sympathetic cardiovascular control

The pre-motor sympathetic neurons arise from areas including the hypothalamus and pons, with the rostral ventrolateral medulla being the primary efferent projection site (Szczepanska-Sadowska et al., 2010). These pre-motor neurons project onto the sympathetic preganglionic cell bodies that form the intermediolateral column in the spinal cord. Postganglionic sympathetic neurons project to multiple organ systems, releasing the catecholamine, noradrenaline (NA), onto the various subtypes of adrenergic receptors found on these tissues (Thomas, 2011). Postganglionic sympathetic fibres innervate the SA and AV nodes to mediate positive chronotropic and dromotropic effects. Sympathetic fibres additionally innervate cardiac ventricular tissue, to exert positive inotropic (contractility) and lusitropic (rate of relaxation) effects (reviewed by Lymperopoulos et al., 2013).

1.3.3.1 Catecholamines and adrenergic receptors

The catecholamines, NA and adrenaline, are involved in sympathetic signalling (Lymperopoulos et al., 2007). NA is synthesised within sympathetic postganglionic nerve terminals, and released onto target adrenergic receptors following neuronal depolarisation (Lymperopoulos et al., 2007). Adrenaline is synthesised and released from the adrenal medulla following sympathetic stimulation, with a smaller amount of NA also produced with way (De Diego et al., 2008; Lymperopoulos et al., 2007). Additionally, recent evidence suggests there may be intrinsic release of catecholamines within the heart from blood vessels and cardiomyocytes (Huang et al., 2005).
Adrenergic receptors are GPCR’s, comprising of $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$, and $\beta_3$ receptor subtypes (Triposkiadis et al., 2009). The endogenous agonists for these receptors, adrenaline and NA, exhibit different affinities for these receptors: adrenaline is slightly more $\beta_2$ selective, whilst NA is more $\beta_1$ selective (Lohse et al., 2003). The receptor subtypes also exhibit different signalling pathways (Table 1). The human heart expresses $\beta_1$: $\beta_2$ in a ratio of approximately 70:30, with $\beta_3$ receptors accounting for a very low proportion (Triposkiadis et al., 2009). Cardiac $\beta_1$ activation leads to positive chronotropic, inotropic, dromotropic, and lusitropic effects, with $\beta_2$ activation playing a smaller chronotropic role (Richter et al., 2008; Triposkiadis et al., 2009). $\alpha_1$-adrenoceptors on arteries, including the aorta and coronary arteries, induce vasoconstriction (Lymperopoulos et al., 2007). $\beta_1$ receptors are coupled to $G_s$ protein, which following agonist binding, leads to adenylyl cyclase activation, subsequent cyclic adenosine monophosphate (cAMP) synthesis, and activation of protein kinase A (PKA) (Triposkiadis et al., 2009). The downstream effects of PKA activation are shown in Figure 1.4: (1) increased intracellular $\text{Ca}^{2+}$ ([Ca$^{2+}$]) through L-type calcium channel (LTCC) and ryanodine receptor (RyR) activation; (2) increased pacemaker activity by hyperpolarisation-activated cyclic nucleotide-gated channel (HCN) activation; (3) increased sarcolemmal $\text{Ca}^{2+}$ reuptake through phospholamban phosphorylation; (4) reduced myofilament $\text{Ca}^{2+}$ sensitivity through phosphorylation of cardiac troponin I (cTnI) and myosin binding protein-C; and (5) increased Na$^+$ influx via phospholemman activation (reviewed by Triposkiadis et al., 2009).
1.3.3.2 Reflexes

Baroreceptor and chemoreceptor afferents project to the nucleus tractus solitarii (NTS) in the medulla. Arterial and cardiopulmonary baroreceptors (stretch receptors) are located in the carotid sinus/aortic arch and atria/pulmonary arteries, respectively. Activation of these receptors in response to increased blood pressure causes a reflex inhibition of efferent sympathetic activity (Thomas, 2011). Chemoreceptors, which are sensitive to arterial partial pressures of oxygen and carbon dioxide (PO₂ and PCO₂, respectively), and pH, are located in the aortic arch so that detection of decreased PO₂/pH or increased PCO₂ by the chemoreceptors leads to sympathetic activation to increase ventilation, with potential synergistic involvement of the baroreflex (Thomas, 2011).

1.4 Cardiac Electrophysiology

The cardiac action potential describes changes in membrane potential according to the flux of the various ions through the cell membrane. The nodal pacemaker cells of the SA and AV nodes possess different physiological parameters to the cardiac ‘work cells’ (Figure 1.5A) (Pinnell et al., 2007). The pacemaker cells possess intrinsic electrical activity and spontaneously depolarise. Depolarisation (phase 0) of the pacemaker cells is primarily induced through calcium entry through T type Ca²⁺ channels, whilst in the work cells is via rapid sodium influx. Repolarisation of both pacemaker cells (phase 3) and work cells (phases
1 – 3) involves potassium efflux, but also calcium influx through LTCC’s in work cells (Pinnell et al., 2007). Following SA nodal discharge, the impulses travel through the atria, before arriving at the AV node. The AV node then slows the impulse, which is transmitted to the interventricular septum, bundle of His, and left and right branches of the ventricles via the Purkinje fibres, leading to regulated contraction of the ventricles (Figure 1.5B) (Pinnell et al., 2007).

The electrocardiogram (ECG) represents the surface cardiac electrical activity, being the sum of all the action potentials in the heart (Figure 1.5B, C) (Klabunde, 2005). The P wave depicts atrial depolarisation and the QRS complex shows ventricular depolarisation. The T wave depicts ventricular repolarisation, with T wave changes shown to be indicative of ischaemia (Klabunde, 2005). The QT interval is the time taken for the ventricles to depolarise and repolarise; prolongation of the QT interval increases the risk of fatal ventricular arrhythmias including torsades de pointes and sudden death (Feldman & Gidal, 2013). Compared to humans, rodents exhibit a short QT interval and lack of isoelectric interval between the QRS complex an T wave (Figure 1.5C), which is attributable to the large outward K+ currents that dominates early ventricular repolarisation (Gussak et al., 2000).

1.5 ANS and Seizures

Seizures are frequently associated with alterations in autonomic function; ictal autonomic symptoms include cardiovascular (heart rate and blood pressure alterations), gastrointestinal (emesis), genitourinary (incontinence), respiratory (apnoea, hyperventilation, neurogenic
pulmonary oedema), and skin and glandular effects (flushing, piloerection, sweating, hypersalivation) (Devinsky, 2004; Moseley et al., 2013). Both partial and generalised seizures are associated with a variety of these changes, arising from seizure activity within central autonomic areas (Devinsky, 2004).

Sevcencu and Struijk (2010) reviewed the literature and found mixed reports of sympathetic dominance, parasympathetic dominance, or suppression of both systems during the interictal period. Typically, signs of sympathetic activation are seen (e.g. tachycardia, increased BP), but parasympathetic symptoms may also arise (e.g. bradycardia, hypoventilation) (Devinsky, 2004). It has been suggested that the sympathetic and parasympathetic symptoms are associated with right and left hemispheric activity, respectively (Sevcencu & Struijk, 2010).

Novak et al. (1999) found temporal lobe seizures were associated with increased sympathetic and decreased parasympathetic activity at seizure onset.

1.5.1 Cardiac changes

Tachycardia is one of the most frequently reported cardiovascular changes during seizures, being reported in 85 – 100% of seizures (Devinsky, 2004; Leutmezer et al., 2003; Mayer et al., 2004; Opherk et al., 2002; Zijlmans et al., 2002). Tachycardia is frequently reported in seizures of right temporal lobe origin, especially in the mesial lobe (Leutmezer et al., 2003; Sevcencu & Struijk, 2010). Acutely, tachycardia induces haemodynamic changes, involving reduced coronary blood flow and cardiac output, and an increased myocardial oxygen demand, increasing the risk of myocardial ischaemia and arrhythmias (Heusch & Schulz, 2007). Bradycardia is considered a rare cardiovascular event in seizures (< 2%), but is serious in that it may progress to asystole (Devinsky, 2004). Bradycardia and asystole are frequently associated with seizures confined to the left temporal region (Leutmezer et al., 2003; Locatelli et al., 1999; Tinuper et al., 2001). Ictal asystole is prevalent in 0.3 – 0.4% of seizures, and has been reported to last up to 60 seconds with potentially fatal consequences, warranting pacemaker implantation in some patients (Rocamora et al., 2003; Sevcencu & Struijk, 2010; Zijlmans et al., 2002). Ictal prolongation of the QT interval is frequently observed during seizures and can increase the risk of arrhythmias and sudden death (Nei et al., 2000; Seyal et al., 2011; Tavernor et al., 1996). Other ECG changes such as AV block, ST depression or T wave inversion are also reported in up to 40% of seizures (Devinsky, 2004; Nei et al., 2000; Tigaran et al., 2003; Tigaran et al., 1997; Zijlmans et al., 2002).

ECG changes observed in various seizure models are comparable to those seen in humans, including tachycardia, bradycardia, increased blood pressure and repolarisation abnormalities
(Bealer & Little, 2013; Bealer et al., 2010; Little & Bealer, 2012; Metcalf et al., 2009a; Metcalf et al., 2009b; Powell et al., 2014; Read et al., 2014).

1.5.2 Respiratory changes
Respiration is controlled centrally via regions in the brainstem, and seizure propagation to these respiratory centres may induce respiratory dysfunction. Tachypnoea, bradypnoea, apnoea, respiratory pauses, and hypoxaemia have been frequently described during seizures (Blum, 2009; O'Regan & Brown, 2005). Furthermore, neurogenic pulmonary oedema and central apnoea and hypopnea have also been discussed as potential causes of SUDEP (Bateman et al., 2010; Johnston et al., 1995; Johnston et al., 1997). The ensuing hypoxaemia and hypercapnia can additionally activate chemoreflex responses (Thomas, 2011).

1.6 Cardiac remodelling
Due to the negligible ability of cardiomyocyte replacement, cardiac remodelling is a crucial process for cardiac repair following injury and involves a number of cell death and inflammatory processes. Whilst cardiac remodelling initially functions to repair the damage and maintain cardiac output, chronic remodelling becomes detrimental and may result in heart failure (HF) (Kapur, 2011). Remodelling may arise from an acute injury (e.g. MI) or from chronic cardiac dysfunction (e.g. volume overload, neurohumoral overactivation) (Mihl et al., 2008). Myocardial oedema is considered a form of reversible ischaemic damage, and has been associated with hypertension and MI (Dongaonkar et al., 2010). Oedema is detrimental to cardiac function through increased myocardial stiffness, impaired systolic and diastolic functions, reduced ventricular compliance, and increased arrhythmia risk (Friedrich, 2010; Mehlhorn et al., 2001).

Cell death is implicated in the pathogenesis of most cardiac diseases, including MI and HF (Chiong et al., 2011). Necrosis is commonly described as a non-regulated form of cell death, involving membrane rupture and the release of cytosolic contents (Chiong et al., 2011). Apoptosis describes a highly regulated form of cell death leading to the phagocytosis of cells without release of harmful substances (Baines, 2011). Apoptosis occurs via the extrinsic (death receptor) or intrinsic (mitochondrial) pathways, which both lead to caspase activation and subsequent apoptosis (reviewed by Konstantinidis et al., 2012). Cardiomyocyte apoptosis rates in the healthy heart are very low, but are shown to be increased in HF and MI (Konstantinidis et al., 2012).

Cardiac inflammatory responses are acutely implicated in the reparative processes occurring following cardiac injury, and excessive inflammation contributes to detrimental remodelling
Neutrophils are recruited early following cardiac injury, followed by monocytes, macrophages and lymphocytes, to clear cell debris (Frangogiannis, 2012). In contrast to necrosis, which elicits an inflammatory response, cells undergoing apoptosis are phagocytosed without the induction of inflammatory processes (Frangogiannis, 2014).

Following the cardiac injury, excessive deposition of collagen by activated fibroblasts is observed, and this is important in maintaining structural integrity of the extracellular matrix (Porter & Turner, 2009). In the heart, collagen I is most prevalent isoform (approximately 85%) (Kong et al., 2014). Reparative fibrosis describes the replacement of infarcted myocardial parenchyma with fibrotic tissue, whilst reactive fibrosis involves the diffuse deposition of interstitial fibrosis subsequent to leukocyte infiltration (Kong et al., 2014). Both interstitial and perivascular collagen deposition can occur, resulting in diastolic dysfunction, impeding myocardial electrical conductivity and reducing coronary vascular compliance (Kapur, 2011; Kong et al., 2014). Fibroblasts are activated in response to various environmental stimuli including stretch, hypoxia, and hormones (e.g. NA and angiotensin II) (Porter & Turner, 2009). Additionally, myofibroblasts, form an activated phenotype in response to pro-inflammatory cytokines (e.g. tissue growth factor-β), hormones (e.g. NA), and mechanical stretch within the diseased tissue, exhibiting increased migratory, proliferative and secretory properties (Porter & Turner, 2009). Myofibroblasts express the contractile protein, α smooth muscle actin (αSMA) (Kong et al., 2014). Unlike fibroblasts however, which undergo apoptosis following resolution of the tissue repair process, myofibroblasts may persist in the infarct scar for months to years, contributing to excessive and pathological remodelling (Porter & Turner, 2009).

1.6.1 Catecholamine-induced cardiotoxicity

Chronically elevated β₁ receptor signalling has been shown to promote cardiomyocyte apoptosis, necrosis, and hypertrophy, leading to cardiac remodelling and HF (Goldspink et al., 2004; Whelan et al., 2013). Mechanisms for these cardiotoxic effects involve intracellular calcium overload, energy substrate depletion, subcellular alterations, and coronary vasospasm (Dhalla et al., 2010). In contrast, β₂ signalling is thought to be protective, as the β₂ receptor can switch coupling to G₁, inducing anti-apoptotic effects (Whelan et al., 2013). Catecholamines may also induce damage independently of adrenergic receptors, via auto-oxidation (Dhalla et al., 2010; Tappia et al., 2001).
1.6.2 Structural cardiac alterations in seizures

Evidence of macroscopic injury, including hypertrophy and dilation, reversible pathology including oedema and irreversible pathology including fibrosis, and inflammatory infiltration has been found in SUDEP cases (Falconer & Rajs, 1976; Natelson et al., 1998; Opeskin et al., 2000; Zhuo et al., 2012). Additionally, evidence of changes to the cardiac conduction system have been seen (Kloster & Engelskjøn, 1999; Opeskin et al., 2000). Cardiac injury following seizure induction is also observed in many of these seizures models, with fibrosis, inflammatory cell infiltration, apoptosis, myocyte vacuolisation, and contraction band necrosis having been observed as early as 48 hours post-seizure induction (Bealer et al., 2010; Metcalf et al., 2009a; Read et al., 2014). Autonomic dysfunction in these models have also been shown, with sympathetic activity increases often observed (Metcalf et al., 2009b; Sakamoto et al., 2008).

1.7 Animal Seizure Models

There are several animal seizure models for investigating specific types of seizures and epilepsies. Genetic seizure models include Stargazer mice (γ2 mutation of voltage-sensitive calcium channel) that model absence seizures, and genetically epilepsy-prone rats that exhibit generalised tonic-clonic seizures (Löscher, 2011; Meldrum, 2002). However, genetic models with channelopathies to induce seizures may also have other effects from channel knockout, including cardiac alterations (Damasceno et al., 2013). Electrical stimulation may be used to induce seizures, which involve electrical stimulation of the whole brain (electroshock seizures), or of a specific region. Electrical stimulation produces increased specificity and control over seizures compared to chemoconvulsant agents, however neuronal damage patterns may not replicate human TLE (Löscher, 2002).

1.7.1 Chemically-induced seizure models

Chemoconvulsant agents may be administered systemically (commonly sc. or ip.) which provide easy and reliable methods of administration, however introduce the possibility of peripheral effects (Leite et al., 2002; Read et al., 2014). Focal application of pro-convulsant substances can also be employed, for example to the amygdala or hippocampus; this models partial seizures, which often spread to secondary generalised seizures (Sarkisian, 2001). Additionally, focal application tends to reduce mortality rate compared to systemic administration (Sharma et al., 2007).

Chemoconvulsants include GABA-related substances that decrease neuronal inhibition (eg. bicuculline) and cholinergic substances such as pilocarpine (muscarinic agonist). Pilocarpine
is a common model for SE and TLE; however, due to pilocarpine’s muscarinic effects (e.g. salivation, tremor), an anti-muscarinic drug is often administered prior which would blunt the parasympathetic system, rendering this a poor model for studying autonomic changes during seizures, especially cardiac changes (Curia et al., 2008). Excitatory amino acid-related substances include KA and domoic acid. These agents have been demonstrated by our group to induce SE and subsequent hippocampal damage (Goulton et al., 2010; Hesp et al., 2007; Read et al., 2014; Sawant et al., 2010)

1.7.1.1 Kainic acid
KA (2-carboxy-4-isopropenyl-pyrrolidin-3-ylacetic acid) is a marine toxin isolated from the red algae *Digenea simplex*. KA is a non-degradable structural analogue of glutamate and is 30 times more potent (Wang et al., 2005). KA has high affinity at KA receptors, 10-fold higher than at AMPA receptors, and produces marked depolarisation of neurons that can elicit excitotoxic effects (Swanson & Sakai, 2009). KA-induced excitotoxicity involves the overactivation of KA and AMPA receptors, and downstream NMDA receptor activation, leading to robust Ca\(^{2+}\) influx and neuronal cell death (Wang et al., 2005).

KA is commonly used as a model to replicate TLE as KA administration induces acute limbic seizure development with histopathological changes including hippocampal sclerosis (Bouilleret et al., 1999; Sperk, 1994). Systemic administration of KA (10 mg/kg, sc.) induces an initial hypomobility period, followed by development of limbic seizures and SE lasting 1–2 hours (Read et al., 2014; Zheng et al., 2010). Up to 90% of rats develop spontaneous recurrent seizures following a latent period of several weeks (Buckmaster, 2004). This is observed in correlation with severe hippocampal damage, especially in the CA3 and dentate gyrus areas (Ben-Ari & Cossart, 2000; Zheng et al., 2010). Nanomolar amounts of KA have also been administered intracerebrally into the ventricle, amygdala and hippocampus (Ben-Ari et al., 1980; Bouilleret et al., 1999; Cavalheiro et al., 1982; Fonnum & Walaas, 1978; Raedt et al., 2009; Sater & Nadler, 1988).

1.8 Antiepileptic drugs and diazepam
Mechanisms of AED’s include sodium channel blockers (e.g. phenytoin, carbemazepine), GABA receptor allosteric modulators (e.g. barbiturates, benzodiazepines), and potassium channel modulators (e.g. retigabine) (Löscher & Schmidt, 2012). AED’s often exhibit much lower half-lives in rats compared to humans due to rapid elimination (Löscher, 2007).

Diazepam (DZP) is a type of benzodiazepine, indicated for anxiety, alcohol withdrawal, muscle spasms, and seizure management (Riss et al., 2008). DZP is commonly used as a first-
line treatment of SE (Babl et al., 2009). Additionally, pre-hospital treatment of SE with DZP has been found to be beneficial in terminating SE and reducing mortality rates (Alldredge et al., 2001). Related to its mechanism at GABA\_A receptors, DZP has several side effects, with hypotension and respiratory depression reported in around 10% of patients (Chapman et al., 2001). Due to its lipophilicity, DZP is rapidly taken up into the brain with equilibrium established within 5 minutes following DZP administration in rats (5 mg/kg, ip.) (Friedman et al., 1986); however, DZP’s lipophilicity also means it is rapidly redistributed to fat stores and has a short duration of action (Chapman et al., 2001). It is shown that a DZP dose of 5 mg/kg (sc.) is effective in attenuating seizures in rats (Baran & Mevissen, 1994; Löscher & Schwark, 1985).

1.9 β-blockers and atenolol

β-adrenergic blockers (β-blockers) are clinically indicated for a range of cardiovascular disorders including angina pectoris, arrhythmias, hypertension, post-MI, and congestive cardiomyopathy (reviewed by Frishman, 2008). β-blockers are broadly divided into lipophilic (e.g. propranolol, metoprolol) and hydrophilic (e.g. atenolol) agents, with hydrophilic drugs exhibiting longer half-lives (Frishman & Saunders, 2011).

Atenolol is a hydrophilic β\_1-selective antagonist. Atenolol’s β\_1 selectively reduces the risk of β\_2-associated respiratory adverse effects (Frishman, 2008). Unlike the lipophilic β-blockers metoprolol and propranolol, atenolol poorly crosses the blood brain barrier (BBB) (Cruickshank et al., 1979). Our laboratory has previously demonstrated atenolol pretreatment (5 mg/kg, sc.) in rats provides effective rate control and cardioprotection following seizures induced by systemic KA administration (Read, 2014).

1.10 Rationale

Autonomic dysfunction during seizures is likely to induce commonly observed cardiovascular changes, such as tachycardia, that may contribute to the increased mortality in epileptics (Devinsky, 2004; Gaitatzis & Sander, 2004). In particular, increased sympathetic activity is likely implicated in the generation of tachycardic arrhythmias, which is detrimental to cardiac structure and function when excessive (Sevcencu & Struijk, 2010). Therefore, there requires a need for pharmacological intervention to reduce the excess sympathetic input to the heart during seizures, and this provides rationale for use of a β-adrenergic antagonist. Atenolol is a selective β\_1 adrenergic antagonist, and has been previously shown in our laboratory to effectively reduce seizure-induced HR changes in rats following prophylactic administration.
(Read, 2014). Additionally, atenolol is currently clinically indicated for rate control, and is hydrophilic and negligibly passes the BBB (Frishman, 2008). However, acute administration of atenolol was not demonstrated to provide neuroprotection following SE in rats, therefore indicating requirement for use of an AED to attenuate seizures and prevent subsequent neurological damage (Bealer et al., 2010). DZP exerts strong anticonvulsant properties and is also indicated for acute seizure management, therefore will be used in this study for attenuating seizures (Alldredge et al., 2001). Seizures will be induced via intrahippocampal KA delivery, which will prevent any potential peripheral effects associated with systemic administration. Pharmacological interventions will be administered at 60 minutes post-seizure induction to replicate a clinical setting, and treatments continued daily for the remainder of the study (to 7 or 14 days) to ongoing continual seizure management.

1.11 Aims and Hypothesis

This thesis aimed to investigate whether pharmacological interventions with atenolol, DZP, or combination treatment of DZP + atenolol, would prevent cardiac damage incurred by KA-induced seizures. It was hypothesised that KA-induced seizures would be associated with acute cardiac changes, potentially including tachycardia and QT prolongation. Saline-treated animals were subsequently expected to demonstrate cardiac injury as evidenced by histological and immunohistochemical methods. It was expected that DZP intervention would attenuate seizures and concurrently would reduce subsequent cardiac alterations and injury. It was also thought that atenolol intervention would attenuate any prevalent tachycardia and would subsequently reduce cardiac injury. Combination therapy with atenolol + DZP was anticipated to effectively attenuate seizure activity and restore normal cardiac function, and therefore provide superior cardioprotection.
2.1 Materials

Animal medicines and drugs were obtained from the University of Otago Animal Welfare Office (Dunedin, New Zealand). Atenolol was obtained from Sigma-Aldrich (Auckland, New Zealand). Kainic acid was purchased from Tocris (Bristol, UK) and dissolved in sterile saline (0.9% NaCl). All other reagents were purchased from Sigma-Aldrich (Auckland, New Zealand) and BDH (Palmerston North, New Zealand) unless otherwise stated.

2.2 Animals

Male Sprague-Dawley rats (320 – 350 g) were sourced from the University of Otago Animal Resource Unit. Animals were housed in a 12 hour light–dark cycle at 22°C with access to standard rat chow and water ad libitum. Animals were allowed 5 – 7 days to acclimatise prior to surgery. Post-surgery, all animals were housed in individual cages. All procedures were performed in accordance with the University of Otago’s Committee on Ethics in the Care and Use of Laboratory Animals and the “Use of Laboratory Animals (NIH Publication No. 85-23, 1996)”. Body weights were measured daily immediately prior to, and then following seizure induction, and soft food and saline (5 ml, sc.) administered as required.

2.3 Experimental Protocol

Animals underwent surgical implantation of intrahippocampal cannulas and telemetric transmitters. Immediately following KA intrahippocampal dosing, animals were randomly assigned into four main drug treatment groups: saline, DZP (5 mg/kg, sc. initial, then 1 mg/kg, sc. bid.), atenolol (5 mg/kg, sc.), or DZP + atenolol combination (using the pre-stated individual doses). Treatments were administered as an intervention at 60 minutes post-KA administration, and continued daily for the remainder of the experiment. Animals from each treatment group were sacrificed at 7 or 14-days post-KA (n = 4 – 8 individual animals per sub-group). All experiments and analysis were carried out in a single-blinded fashion. Intervention drug doses were derived using human-to-animal conversions via body surface area calculation (Reagan-Shaw et al., 2008).

2.4 Implantation of intrahippocampal cannulas and telemetric transmitters

Intrahippocampal cannulae insertion and telemetry implantation surgeries were performed by PhD student Morgayn Read and MSc student Dominic McCann. Antibiotics (amphotericin, 60 mg/ml, 0.2 ml, sc.) and pain relief (carprofen, 5 mg/kg, sc.) were administered 30 minutes prior to surgery and daily for 3 days post-surgery. Animals were anaesthetised with ketamine hydrochloride (75 mg/kg, sc.) and domitor (medetomidine hydrochloride, 0.5 mg/kg, sc.) with
atropine (0.05 mg/kg, sc.). Animal body temperatures were maintained at 37°C through the surgery using a heating pad (Harvard Apparatus, Holliston, MA, USA). Implantations of transmitters were performed as previously described (Goulton et al., 2010; Read et al., 2014). Briefly, a two-channel (ECG and electroencephalograph [EEG]) telemetric transmitter (Telemetry Research, Auckland, New Zealand) was inserted into a subcutaneous pocket in the lower abdomen. The ECG reference electrode was sutured to the xiphoid process and the recording electrode was sutured close to the right atrium. EEG electrodes were tunneled subcutaneously and positioned using a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA), as previously described (Sawa et al., 2010). The coordinates for the electrodes and cannula are described in Figure 2.1.

<table>
<thead>
<tr>
<th></th>
<th>Bregma</th>
<th>Midline</th>
<th>Depth from skull surface</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recording electrode</td>
<td>~5.2 mm</td>
<td>5 mm to left</td>
<td>2.0 mm</td>
</tr>
<tr>
<td>Reference screw</td>
<td>+2.0 mm</td>
<td>2 mm to right</td>
<td>1.0 mm</td>
</tr>
<tr>
<td>Hippocampal cannula</td>
<td>~5.2 mm</td>
<td>5 mm to right</td>
<td>5.2 mm</td>
</tr>
</tbody>
</table>

Figure 2.1: Table on left showing skull coordinates of electrodes and cannula placement. Image on right shows placement of the reference and recording EEG electrodes, and the intrahippocampal drug delivery cannula at the coordinates shown in Table on left. Image adapted from Luckl et al. (2010).

Holes were burred into the skull using the co-ordinates mapped out with aid of the stereotaxic frame, and a drug cannula (26G) placed to allow KA delivery into the dorsal hippocampus. EEG electrodes and cannulae were fixed in place with dental cement, and the abdomen and scalp wounds closed using subcutaneous 5-0 braided silk sutures. Localised anaesthetic relief was provided using lignocaine hydrochloride (6 mg/ml) at the wound sites and lignocaine gel (2%) applied post-recovery. Domitor sedation was reversed with Antisedan™ (atipamezole hydrochloride, 0.5 mg/kg, sc.). In this study, 7-day animals were implanted with both cannulae and transmitters, whereas the 14-day animals were only implanted with the drug cannulae, as rat growth over the 14-day period exceeded the length and extension of the recording leads. Animals were housed individually and allowed to recover for 7 days prior to seizure induction.

2.5 Seizure induction and behavioural/telemetric recordings

Animals were habituated daily to being comfortably restrained in a tea towel to reduce handling stress artefacts during the KA administration and subsequent drug administration protocols. Animals were placed in a Perspex chamber (1 m x 50 cm x 50 cm; Aburns Glass, Dunedin, New Zealand) for 30 minutes prior to seizure induction for acclimation. ECG, EEG
and behavioural data were simultaneously recorded for a 30-minute baseline period prior to KA administration, and continued for 3 hours post-KA. Telemetric data was sampled at 2 kHz using a Powerlab 2/25 signal conditioner and LabChart v.6 software (ADIInstruments, Dunedin, New Zealand). Seizures were induced by administration of KA (2 nmol in 1 µl sterile 0.9% NaCl) via the intrahippocampal cannula, using a Hamilton glass syringe (Hamilton, Reno, NV, USA) and automated infusion pump (BASi, West Lafayette, IN, USA) at a rate of 1 µl/min for 1 minute (Sawant et al., 2010). Animal seizure behaviour was recorded every 15 seconds and scored using a 5-point scale, adapted from Hesp et al. (2007) and Racine (1972) (Table 2). Telemetric and behavioural data were also recorded for 60 minutes at 24 hours, 48 hours, and 7 days post-KA administration.

### Table 2: Levels of seizure behaviours, with examples of behaviours at each level. Adapted from Hesp et al. (2007).

<table>
<thead>
<tr>
<th>Level 0</th>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
<th>Level 4</th>
<th>Level 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Discomfort</td>
<td>Mild seizure</td>
<td>Moderate</td>
<td>Severe generalised</td>
<td>Tonic-clonic</td>
</tr>
<tr>
<td>behaviours</td>
<td>behaviours</td>
<td>behaviours</td>
<td>seizure behaviours</td>
<td>seizures</td>
<td>convulsions</td>
</tr>
<tr>
<td>Walking</td>
<td>Blinking</td>
<td>Freezing</td>
<td>Wet dog shakes</td>
<td>Loss of balance</td>
<td>Loss of balance</td>
</tr>
<tr>
<td>Resting</td>
<td>Abnormal resting</td>
<td>Mastication</td>
<td>Salivation</td>
<td>Rearing</td>
<td>Rearing</td>
</tr>
<tr>
<td>Sniffing</td>
<td>Heavy breathing</td>
<td>Head bobbing</td>
<td>Circling</td>
<td>Foaming</td>
<td>Foaming</td>
</tr>
<tr>
<td>Wall climbing</td>
<td>Hiccups</td>
<td>Head shakes</td>
<td>Forelimb clonus</td>
<td>Myoclonal jerks</td>
<td>Myoclonal jerks</td>
</tr>
</tbody>
</table>

#### 2.6 ECG recordings

ECG data were recorded using LabChart v.6 Pro ECG software (ADIInstruments, Dunedin, New Zealand) and HR, corrected QT (QTc) interval, and T wave amplitude analysed using the dedicated software modules. QT intervals were corrected for changes in HR using the Mitchell analysis algorithm \[ QTc = QT/(RR/100)^{0.5} \] (Mitchell et al., 1998). Data were analysed using averaged 1 minute data blocks every second minute during the initial 3.5-hour study, and over 30-minute blocks at the 24-hour, 48-hour and 7-day studies. EEG data was separately analysed by PhD student Morgayn Read to confirm the occurrence of seizure activity in the brain (Read, 2014).

#### 2.7 Oxygen saturation

Oxygen saturation (SpO₂) was recorded using an oximeter pod (ADIInstruments, Dunedin, New Zealand). A sensor clip was attached to the base of the tail and the dedicated LabChart v.6 Pro software used to analyse animal oxygen saturation levels. SpO₂ levels were recorded pre-KA (baseline), at 1-, 3-, 24-, 48-hours, and 7- and 14-days post-KA.
2.8 Blood pressures

BP’s were recorded in a small subset of animals ($n = 2 – 4$). Blood pressures were recorded using a non-invasive pulse transducer/pressure cuff and PowerLab system (AD Instruments, Dunedin, New Zealand), and analysed using the LabChart Blood Pressure module (AD Instruments, Dunedin, New Zealand). BP’s were collected pre-KA (baseline), at 1-, 3-, 24-, 48-hours, and 7- and 14-days post-KA, and the average of three repeat readings were used.

2.9 Cardiac troponin I

Tail vein blood samples were collected for blood chemistry 1 day prior to KA administration (baseline), and at 24- and 48-hours, and 7- and 14-days post-KA. Samples were centrifuged at 3000 r.p.m. for 3 minutes, and the plasma collected and stored at -80°C until analysed. Cardiomyocyte myofilament damage was assessed by measuring plasma levels of cTnI levels using a high sensitivity rat cardiac troponin-I ELISA kit (Life Diagnostics Inc., West Chester, PA, USA), according to the manufacturer’s instructions. Briefly, 100 µl of standards (0 – 10 ng/ml cTnI) and plasma samples (diluted 1:4) were added in duplicate to individual wells in a 96-well plate containing 100 µl of the cTnI horseradish peroxidase (HRP) conjugate. The plate was mixed at room temperature (RT) for 60 minutes on an orbital shaker, before wells were washed thoroughly with the wash solution provided in the kit. Tetramethylbenzidine reagent (100 µl) was added to each well and left to incubate for 20 minutes at RT. The kit Stop Solution was then added to each well and mixed gently to observe a colour change from blue to yellow. End point absorbances were read at $\lambda = 450$ nm using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA), and sample cTnI concentrations calculated from the generated standard curve.

2.10 Sacrifice of animals and heart processing

At 7- or 14-days post-seizure induction, animals were euthanised under halothane sedation in an induction chamber. A final blood sample was taken from the vena cava and a thoracotomy rapidly performed. The heart was excised, and the aorta cannulated using a Langendorff rig to allow retrograde-perfusion with saline (0.9% NaCl, 4°C). Hearts were arrested in diastole with 20 mM potassium chloride. The tissue was perfused-fixed with 10% neutral-buffered formalin (Thermo Fisher Scientific, Albany, New Zealand) and left to fix in the solution overnight at 4°C. Hearts were then stored in 70% ethanol at 4°C until embedding. For embedding, three transverse sections were obtained per heart at 2, 4, and 6 mm from the apex (Figure 2.2) using a rat heart matrix (Zivic Instruments, Pittsburgh, PA, USA). Heart sections
were dehydrated in sequentially increasing concentrations of ethanol, and paraffin-embedded and sliced at 4 µm onto Dako FLEX IHC slides (Dako, Glostrup, Denmark). This sectioning was performed by the University of Otago Histology Department. Hearts from sham control animals were also processed for immunohistochemistry (n = 4).

Figure 2.2: Figure demonstrating how each heart was sectioned for immunohistochemistry processing. Three sections were cut at 2, 4, and 6 mm from the apex (left), representing labelled sections A, B, and C, respectively (right). Throughout this thesis these sections will be referred to as the apical, mid-ventricular, and basal-ventricular sections, respectively. RA: right atrium; LA: left atrium; RV: right ventricle; LV: left ventricle.

2.11 Immunohistochemistry

2.11.1 ApopTag

The commercially available apoptosis marker kit ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA, USA) was used to detect apoptotic cells in paraffin-embedded sections. This procedure is based on the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay, which involves the labelling of 3-OH ends of DNA strand breaks of apoptotic cells (Walker & Quirke, 2001). The protocol used was based on the manufacturer’s instructions and previous relevant publications (Lopez-Candales et al., 1997; Mensah-Brown et al., 2002), and optimised for specific use in our tissue. Slides were deparaffinised in xylene for 10 minutes and rehydrated in a series of ethanol dilutions (2 × 100%, 95%, 2 × 85%, 50%; 4 minutes each). Following incubation in phosphate-buffered saline (PBS; pH 7.4) for a further 4 minutes, proteinase K (20 µg/ml) was applied to the slides for 20 minutes at RT followed by 2 × 2 minute washes in double-distilled water (ddH2O). Peroxidase activity was blocked with 3% H2O2/PBS for 10 minutes, followed by 2 × 5 minute PBS washes. Equilibration buffer (17.5 µl/slide) was applied, coverslipped and incubated for 15 minutes at RT in a fumehood. The equilibration buffer was then carefully blotted off and terminal deoxynucleotidyl transferase (TdT) enzyme applied (12.5 µl/slide) at a working-strength (114 µl reaction buffer with 36 µl TdT enzyme). The coverslipped sections were then incubated for 30 minutes in a humidified chamber at 37°C. TdT enzyme was then tapped off and pre-warmed Stop-Wash buffer applied and incubated for 30 minutes at 37°C, with gentle agitation every 10 minutes. Slides were washed in 3 × 2 minute PBS washes, and HRP-
labelled anti-digoxigenin supplied with the kit was added (17.5 µl/slide with coverslip) and left for 30 minutes at RT. Slides were rinsed in 3 × 5 minute PBS washes. HRP-labelled anti-digoxigenin was visualised using 3,3’-diaminobenzidine (DAB; Vector Laboratories, Burlingame CA, USA), for 10 minutes. Slides were washed in ddH₂O for 5 minutes, and nuclei counterstained with Gill’s No.2 haematoxylin (Histology Department, University of Otago, Dunedin, New Zealand) for 10 seconds, then rinsed thoroughly in running water for 5 minutes. Finally, slides were dehydrated in a series of ethanol dilutions (50%, 2 × 85%, 95%, 2 × 100%) for 3 minutes each, cleared in xylene for 30 seconds, and coverslipped with dibutyl phthalate xylene (DPX) mountant. A negative control was carried out by replacing TdT enzyme with ddH₂O.

2.11.2 CD68
Slides were deparaffinised in xylene for 10 minutes and rehydrated in a graded series of ethanol (100%, 95%, 85%, 50%; 3 minutes each) and PBS for 5 minutes. A hydrophobic barrier was drawn around tissue sections using a Dako Pen (Dako, Glostrup, Denmark). Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) for 30 minutes at 95°C, and cooled at RT for 20 minutes. Following 2 × 5 minute washes in PBS, animal-free blocker (diluted 1:5 in ddH₂O; Vector Laboratories, Burlingame, CA, USA) was applied for 2 hours at RT. Slides were then washed for 10 minutes in 1% bovine serum albumin (BSA)/PBS. The primary antibody CD68 (clone ED1; ab31630, Abcam, Cambridge, UK) was diluted 1:100 in 1% BSA/PBS and incubated at 4°C overnight. Following 3 × 5 minute PBS washes, endogenous peroxidases were blocked with 0.3% H₂O₂/PBS for 10 minutes. Sections were rewashed (3 × 5 minutes) and incubated with the secondary antibody (HRP-labelled goat anti-mouse IgG, 1:500; Pierce, Rockford, IL, USA) for 90 minutes at RT. Subsequently, slides were thoroughly washed in PBS. DAB was used to visualise staining; slides were incubated in DAB for 12 minutes in the dark before being rinsed in ddH₂O for 5 minutes. Slides were counterstained in Gill’s haematoxylin for 10 seconds, rinsed thoroughly in water, dehydrated and coverslipped using DPX. A positive control for CD68 was carried out on formalin-fixed paraffin-embedded rat spleen tissue. A negative control was carried out by omitting the primary antibody.

2.11.3 Collagen I
Protocols for collagen I staining are as described for CD68 staining, with a slightly amended protocol to optimise staining for this isotope. Following deparaffinisation in xylene and rehydration in ethanol, heat-induced epitope retrieval was performed in 10 mM citrate buffer
(pH 6.0) for 20 minutes at 95°C and cooled at RT for 20 minutes. Following 3 × 3 minute washes in ddH$_2$O, an additional enzymatic digestion using hyaluronidase was included to improve epitope retrieval as initial trials with this double antigen retrieval protocol appeared to increase staining intensity. Hyaluronic acid is found in the extracellular matrix and may mask extracellular epitopes such as collagen, thus hyaluronidase treatment can unmask these (D'Amico et al., 2009). For this step, tissue sections were first acidified in 100 mM Tris-HCl (pH 5.5) for 2 changes of 10 minutes each, followed by incubation with bovine testicular hyaluronidase (2 mg/ml in 100 mM Tris-HCl, pH 5.5; Sigma-Aldrich, St Louis, MO, USA) for 1 hour at 37°C. Tissue pH was then neutralised with 3 × 5 minute washes in PBS. Non-specific blocking of epitopes was conducted using an animal-free blocker as described above with CD68. Collagen type-I antibody (polyclonal rabbit; catalogue #600-401-103-0.5, Rockland, Gilbertsville, PA, USA) was diluted 1:100 in 0.1% BSA/PBS. The secondary antibody (goat anti-rabbit IgG-HRP; sc-2030, Santa Cruz Biotechnology, Dallas, TX, USA) was diluted to 1:400 in 0.1% BSA/PBS. DAB was applied for 2 minutes. A negative control was carried out by omitting the primary antibody.

2.11.4 αSMA
Immunohistochemical staining for αSMA was performed as for CD68, with some amendments to the protocol. Antigen retrieval was not performed as it was found to result in non-specific nuclear staining. The primary antibody (αSMA, A5228, Sigma-Aldrich, St Louis, MO, USA) was diluted at 1:400 with 0.3% Triton X-100 in 0.1% BSA/PBS and incubated onto the tissue sections at 4°C overnight. Triton X-100 was used as a permeabilisation agent as αSMA is a cytoplasmic protein. The secondary antibody used was an HRP-labelled goat anti-mouse IgG (1:2000, Pierce, Rockford, IL, USA), diluted in the same diluent as the primary and incubated onto the sections for 90 min at RT. DAB was applied for 5 minutes. A negative control was carried out by omitting the primary antibody.

2.12 Digital analysis of immunohistochemistry
Whole slide images were captured at 20 × (objective power) using the Aperio ScanScope CS2 digital scanning system (Aperio Technologies, Vista, CA, USA). The software packages ImageScope (Aperio Technologies, Vista, CA, USA) and Photoshop CS3 (Adobe, San Jose, CA, USA) were used for digital analysis, as described below.
2.12.1 Interstitial space

Interstitial space was analysed on ApopTag-stained sections. This quantification not only took into account interstitial oedema, but also blood vessels. Screenshots of each full ventricle level were captured and imported into a blank Photoshop file. Using the magnetic lasso tool, the outside of the heart was traced and ‘layered via cut’. The same procedure was done to remove the ventricle chambers. Subsequently, only the tissue was selected and the magic wand tool was used to select the background colour of interest, with the colour tolerance set to 10. The number of selected pixels was recorded from the Histogram toolbar, and this was calculated as a percentage of the total pixels of that layer. These values were recorded in an Excel spreadsheet (Microsoft Office 2010, Redmont, WA, USA).

2.12.2 ApopTag analysis

ApopTag staining was analysed manually using Aperio images captured at 20 × magnification. The images were viewed with ImageScope and the total number of positive nuclei were manually counted across each full ventricle layer and expressed as a raw value.

2.12.3 CD68 analysis

The Aperio ImageScope Nuclear Count v.9 algorithm was used for quantifying CD68 staining. Although CD68 is a membrane marker, the Membrane Quantification algorithm attached to Image Scope was found to be ineffective in picking up these cells, therefore the Nuclear algorithm was applied. Positive nuclei were quantified and averaged across 10 random fields at 10 × magnification for the basal- and mid-ventricle levels, and 6 fields for the apical level. The algorithm analysed nuclei according to their positive hue threshold, with positive nuclei being labelled as strong-positive, positive, or weak-positive (Figure 2.3). The total number of positive nuclei were totalled per 10 × field, and divided by the area of the field to attain a quantitative value of positive cells per mm².
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2.12.4 Collagen I analysis

Analyses were carried out separately on each ventricular level, with 10 random fields analysed at each of the basal- and mid-ventricle levels, and 6 fields at the apical level. The field images were captured from the Aperio images at 10 × magnification, and were imported into Photoshop. Semi-quantitative analysis of DAB staining was conducted on the images using Photoshop CS3 (Adobe, San Jose, CA, USA) and calculated as the amount of collagen I positive-pixel counts. Briefly, the magic wand tool in Adobe Photoshop was used to select the positively DAB-stained brown colour, and the tolerance set to 50 to increase the range of colours surrounding the selected hue. The number of selected pixels was detailed in the Histogram box, and this was recorded. The background colour outside of the tissue region was subsequently selected, with tolerance set to 10 and this number of selected pixels also noted. The total number of pixels contained within each 10 × image field was constant for all images. The total stained area (%) was calculated by the following equation:

\[
\frac{\text{(Total positive pixels)}}{\text{(Total image pixels} - \text{Total background pixels})}\times 100
\]

This equation accounted for any discrepancies in interstitial space (oedema and blood vessels). These values were then averaged for each level.

2.12.5 αSMA analysis

The Positive Pixel v.9 Algorithm on Scanscope software (Aperio Technologies, Vista, CA, USA) was used to quantify the number of positively stained pixels across each full ventricle level. The default settings for DAB staining were used. Following analysis, the program calculated the proportion of positive pixels for each level.
2.13 **Statistical analysis**

Statistical analysis was performed using Prism™ v.5 (GraphPad, San Diego, CA, USA). Behavioural data was analysed as the maximum score attained per minute, and the total number of WDS, Level 4 and Level 5 behaviours per minute. Behavioural data was analysed using a non-parametric 2-way Kruskal-Wallis ANOVA. ECG data, SpO₂, body weights, blood pressures, cTnI levels and immunohistochemistry data were analysed using a parametric 2-way ANOVA with Bonferroni post-hoc. Results are displayed as mean ± SEM. Statistical significance was set at a level of $P < 0.05$. 
3.1 **Behavioural scores**

KA (2 nmol) administration induced high-level seizures, with seizure levels significantly increased above baseline within 10 minutes ($P < 0.05$ vs. baseline in all groups). All animals reached comparable seizure levels within the first 60 minutes in regards to cumulative score, total WDS and total Level 4 behaviours (Figures 2.1B-D), however one animal reached Level 5 seizures in the atenolol group prior to intervention (Figure 2.1E; $P < 0.05$ vs. saline). Animals who did not achieve observable seizures within 60 minutes, either via observation or by EEG analysis when available, were presumed to not have received appropriate KA delivery and were omitted from the study ($n = 3$). Saline and atenolol interventions at 60 minutes post-KA did not have any effect on seizure scores, and the average seizure scores remained elevated above baseline up to 24 hours in these groups ($P < 0.05$; Figure 2.1A). DZP and combination interventions were very effective in reducing the average seizure scores below saline animals within 10 minutes ($P < 0.05$), and these treatments reduced behaviours to baseline levels within 30 minutes of administration. DZP and combination interventions also significantly reduced cumulative seizure score, total WDS, and total Level 4 behaviours (Figures 2.1B-D; $P < 0.05$ vs. saline).

3.2 **Morbidity and Mortality**

One rat died at 30 minutes following atenolol intervention (i.e. 1.5 hours post-KA), however this may have been due to an underlying factor, as another rat from the same litter but involved in a separate study was also noted to have died within the initial seizure period. Additionally, a combination-treated animal died suddenly during handling at 4 days post-KA, following the spontaneous development of Level 4 – 5 seizures, and this was attributed to possible SUDEP. One DZP-treated rat was euthanised at 5 days post-KA, following the development of suspected acute HF, with the rat exhibiting severe ECG changes, peripheral oedema and ascites.
Figure 3.1: Seizure behaviours following KA (2 nmol). A: Average maximal seizure levels attained, averaged across every 10 minutes. KA was administered at $t = 0$ and pharmacological intervention at $t = 60$ minutes. B–E: Behavioural scores demonstrating cumulative seizure scores, B; total wet dog shakes, C; total level 4 behaviours, D; and total level 5 behaviours, E. Total time blocks were 30 min baseline, 1 hour period post-KA, 2 hour period post-intervention, and 30 min blocks at 24 hours, 48 hours, and 7 days post-KA administration. Data shows mean ± SEM. (n = 5 – 7 per group) * $P < 0.05$ vs. saline treatment.
3.3 **Body weights**

Body weights were normalised to baseline (day 0) to account for any variations in starting body weight. Animals tended to lose weight in the first 24 hours following seizure induction; the saline group body weights dropped by 8.6 ± 0.01% of baseline (Figure 3.3), however the combination group lost significantly less weight compared to the saline group (1.2 ± 0.01%; *P* < 0.05 vs. saline). All animals recovered this lost weight by 48 hours. Animals steadily put on body weight for the rest of the study, although the saline group exhibited a small weight drop on day 11 post-KA. The combination group exhibited significantly heavier body weights at days 13 and 14 compared to the saline group (1.32 ± 0.03 vs. 1.21 ± 0.02 of baseline; *P* < 0.05).

*Figure 3.2: Daily body weights normalised to baseline (day 0). KA (2 nmol) was administered on day 0 and animals were sacrificed at day 7 or 14. Shows mean ± SEM (n = 4 – 12 per time point) * P < 0.05 vs saline group.*
3.4 ECG data

Figure 3.3: ECG data demonstrating: A, heart rate (HR) in beats per minute (b.p.m.); B, corrected QT (QTc) interval; and C: T wave amplitude. A 30 minute baseline period was recorded, KA (2 nmol) was administered at $t = 0$, and pharmacological intervention given at $t = 60 \text{ min post-KA}$. Data shows mean ± SEM across 1 minute blocks, every other minute, with 30 minute blocks averaged at 24 h, 48 h, and 7 days post-KA. ($n = 5 – 7 \text{ per group}$). * $P < 0.05 \text{ vs. saline treatment}$.
3.4.1 Heart rate

Baseline HR’s prior to KA administration ranged from 354.1 ± 18 – 377.1 ± 23 b.p.m. (Figure 3.3A). Following KA administration, HR’s rapidly increased up to 482.3 ± 15 b.p.m. within 2 minutes. HR’s remained significantly elevated in all groups above their respective baselines from 2 – 60 minutes post-KA ($P < 0.05$), with one rat reaching a peak HR of 588.0 b.p.m., prior to combination intervention. In the saline group, HR’s remained elevated above baseline up until 180 minutes ($P < 0.05$), but had returned to baseline rates by 24 hours. HR’s of DZP-treated animals remained elevated above baseline up to 48 hours post-KA ($P < 0.05$). Moreover, DZP-treated animal’s HR’s were significantly increased above saline animals at 24 and 48 hours. Atenolol and combination treatments rapidly reduced HR’s to baseline levels within 2 minutes, and HR’s remained at baseline levels for the full 7 day-study. Additionally, HR’s in the atenolol and combination groups were significantly reduced against saline-treated animals from 68 minutes through until 7 days ($P < 0.05$).

3.4.2 QTc interval

QTc intervals were normalised to baseline values to account for baseline variations between animals. Following KA administration, QTc intervals immediately increased and remained prolonged through the first 60 minutes ($P < 0.05$ vs. baselines; Figure 3.3B). QTc intervals remained prolonged until 7 days in the saline-treated group and 48 hours in the DZP-treated group ($P < 0.05$ vs. respective baseline). Atenolol and combination interventions rapidly reduced QTc intervals to baseline levels by $t = 64$ minutes post-KA and these remained at baseline levels throughout the remainder of the study (to 7 days). When compared to saline QTc intervals, atenolol and combination QTc intervals were only significantly shorter than
saline values at several time points, however both were consistently lower than saline at 24 hours, 48 hours, and 7 days ($P < 0.05$ vs. saline).

3.4.3 **T wave amplitude**

T wave amplitudes were normalised to baseline values to account for variations in proximity of recording electrodes to the right atria. There were no significant differences in T wave amplitudes during the study recording periods compared to saline values (Figure 3.3C). However, T wave amplitude was decreased by 51% below baseline in the DZP group at 7 days post-KA ($P < 0.05$ vs. baseline).

3.5 **Blood pressure**

Baseline BP’s ranged from $81.0 \pm 0$ mmHg to $99.1 \pm 0.4$ mmHg, and there were no observed differences between groups (Figure 3.5A). At both 1 and 3 hours post-KA, DZP-treated rats ($n = 2$) had significantly increased BP’s above their respective baseline, and against the saline group (both $P < 0.05$). Of note, one DZP animal experienced an 88% increase above its baseline BP at 3 hours post-KA, to 186.1 mmHg. Blood pressures in the saline group were also significantly increased to $119.6 \pm 6.7$ mmHg at 48 hours ($P < 0.05$ vs. baseline). All other BP values demonstrated no difference to each group’s respective baseline value. However it should be noted these are preliminary results as the sample size was very small ($n = 2 – 4$).

3.6 **Oxygen saturation**

Baseline SpO$_2$ ranged from $91.7 \pm 2$ to $95.9 \pm 1$ % (Figure 3.5B). There were no significant differences compared to saline animals at any time points. However, at 3 hours post-KA, SpO$_2$ values were reduced to $88.4 \pm 0.7$ % and $88.6 \pm 3.0$ % in the DZP-treatment and atenolol-treatment groups, respectively ($P < 0.05$ vs. respective baselines). Of note, all atenolol-treated rats at 14 days ($n = 4$) demonstrated SpO$_2$ values of 97%.
Figure 3.5: A: Systolic blood pressures were measured via a tail cuff at various time points following KA administration. (n = 2 – 4) per group. B: Blood oxygen saturation (SpO₂) measured at various time points following KA, (n = 4 – 8). * P < 0.05 vs. saline group. # P < 0.05 vs. baseline.

Figure 3.6: Cardiac troponin I levels (cTnI) were measured by a cTnI plasma ELISA kit from tail vein samples collected 24 hours prior to KA administration, and subsequently at 24 h, 48 h, 7 days, and 14 days post-KA administration. (n = 5 - 6 per group). # P < 0.05 vs. respective baseline.
3.7 Cardiac troponin I levels

Baseline troponin levels were found to range from 0.025 ± 0.01 to 0.049 ± 0.01 ng/ml (Figure 3.6), and these values are in accordance with other studies (Bertinchant et al., 2000; York et al., 2007). At no time point were cTnI values significantly different compared to saline-treated animal levels. At 24 hours post-KA, troponin levels had increased 4.2-fold above baseline in the saline group to 0.206 ± 0.04 ng/ml ($P < 0.05$ vs. baseline). Similarly, levels were significantly increased in the DZP group (0.229 ± 0.08 ng/ml; $P < 0.05$ vs. baseline). Cardiac troponin I levels also appeared increased in the combination group at 24 hours but this was not significant. Subsequent troponin levels at 48 hours, 7 days and 14 days were found to be non-significant compared to baseline values. There were also no significant differences between groups at any of the time points measured.

3.8 Immunohistochemistry and histology

3.8.1 Interstitial space

Interstitial space was measured by calculating the percentage of background visible, and consequently this value also includes vessel lumens. Oedema was often localised within the subendocardial regions (Figures 3.7B-C). Saline- and DZP-treated rat hearts showed increased interstitial space at both 7- and 14-days post-KA, in all three ventricle levels (Figures 3.7F-G; $P < 0.05$ vs. control). The only observed difference between 7- and 14-day interstitial space levels was a reduction at 14-days in the apical section of DZP-treated hearts (36.9 ± 2.8 vs. 25.4 ± 2.4 %; Figure 3.7G; $P < 0.05$). Additionally, there was significantly more interstitial space in the DZP hearts of apical sections compared to basal ventricle sections.

3.8.2 Apoptag

Apoptotic cells were found diffusely throughout the ventricle (Figure 3.7C). Apoptotic cells also often appeared to show morphology similar to that of macrophages and fibroblasts (Figure 3.7D). The basal-ventricular section showed 7.0 ± 2 apoptotic cells in control hearts (Figure 3.8G). Apoptosis levels were significantly increased up to 15-fold against control levels in both saline- and DZP-treated hearts, at both 7- and 14-days (Figures 3.7G-I; $P < 0.05$ vs. control). Apoptotic cell counts in atenolol- and combination-treated rat hearts were not increased against control levels. Comparable results were seen in the mid-ventricle section (Figure 3.7H). In the apical section, 7-day rat hearts in both saline and DZP-treated rats were significantly increased compared to controls, however at 14 days only saline-treated hearts
were increased above controls ($P < 0.05$), whilst atenolol- and combination-treated hearts were significantly reduced against saline-treated hearts ($P < 0.05$).

### 3.8.3 CD68

CD68$^+$ cells were often diffuse throughout the myocardium, although infarcts were also seen (Figures 3.9D-E). Figure 3.9E shows a small infarct area in a 7-day DZP-treated rat, with myocardial CD68$^+$ cell infiltration, whilst Figure 3.9H shows intense CD68$^+$ infiltration in the subendocardial region of a rat that died 30 minutes post-atenolol intervention. Quantification of CD68$^+$ cells found significant increases in saline- and DZP-treated rat hearts in the basal- and mid-ventricle sections, with saline-treated rat hearts showing values of 84.0 ± 8 and 89.5 ± 11 cells/mm$^2$ at 7 and 14 days, respectively (Figures 3.9I-J; both $P < 0.05$ vs. control). In all 7-day rat hearts in both atenolol and combination groups, CD68 levels were reduced to control levels, although this observation was not as striking in 14-day animal hearts. There was a significant reduction in the number of CD68$^+$ cells/mm$^2$ in the saline-treated rat hearts in the apical section compared to the basal-ventricle section (Figure 3.9K; $P < 0.05$).

### 3.8.4 Collagen I

Collagen I deposition was located both perivascularly and interstitially (Figures 3.10C-D). Surprisingly, a 7-day combination-treated rat was found to have an infarct area with extensive collagen I deposition (Figure 3.11C). Collagen I was significantly increased above controls in saline- and DZP-treated rat hearts at 7 days in both basal and mid-ventricular sections ($P < 0.05$; Figure 3.10G-H). Atenolol- and combination-treated hearts showed a significant reduction in collagen deposition against saline-treated hearts in basal and mid-ventricular sections ($P < 0.05$). Results in the apical section were less significant, with only DZP hearts showing an increase over control levels, to 47.0 ± 3.2% oedema ($P < 0.05$, Figure 3.10I). There were no differences between 7- and 14-day hearts, nor any differences between the 3 ventricle levels between any groups.

### 3.8.5 αSMA

There were no observed significant differences between groups in αSMA expression in all ventricular levels (Figures 3.11D-F). There were also no differences between 7 and 14-day hearts in any groups, nor any differences αSMA expression between ventricular levels. In the combination-treated rat that demonstrated an infarct with collagen I deposition, αSMA staining was also found in this area signifying the presence of myofibroblasts (Figure 3.11B-C).
Figure 3.7: Myocardial oedema histology analysis. Figures A – E show representative images of myocardial oedema (interstitial space): (A) naïve control; (B) saline-treated; (C) diazepam (DZP)-treated; (D) atenolol-treated; and (E) DZP + atenolol-treated seizure animals. Images were captured at 20 x magnification and scale bars represent 100 µm. Graphs F - H demonstrate quantification of total percentage interstitial space across whole transverse ventricle sections at the basal-, mid- and apical-levels of the ventricle, respectively. * P < 0.05 vs. control; # P < 0.05 vs. saline-treated group. Each data point represents mean ± SEM. (n = 4 animals per group).
Figure 3.8: ApopTag immunohistochemistry of cardiac sections. Figures A – F show representative images of ApopTag: (A) negative control; (B) naive control; (C) saline-treated; (D) diazepam (DZP)-treated; (E) atenolol-treated; and (F) diazepam + atenolol-treated seizure animals. Images were captured at 40 x magnification and scale bars represent 100 µm. Arrows depict apoptotic cells. Graphs G - I demonstrate quantification of total ApopTag-positive cells in each transverse ventricle sections at the basal-, mid- and apical-levels of the ventricle, respectively. Sections were stained with DAB and counterstained with Gill’s haemotoxylin. * P < 0.05 vs. control; # P < 0.05 vs. saline-treated group. Each data point represents mean ± SEM. (n = 4 animals per group).
Figure 3.9: Macrophage CD68 immunohistochemistry of cardiac sections. Figures A – G show representative digital images of CD68 staining, with arrows depicting CD68-positive cells: (A) negative control in spleen; (B) positive control in spleen; (C) naïve control; (D) saline-treated; (E) diazepam (DZP)-treated; (F) atenolol-treated; and (G) DZP + atenolol-treated seizure animals. Figure H shows intense subendocardial CD68 infiltration in an atenolol-treated animal that died 1.5 hours post-KA. Figures A – G were captured at 40 x magnification and Figure H was captured at 20 x magnification. Scale bars represent 100 µm. Graphs I - K demonstrate quantification of CD68 staining in each transverse ventricle sections at the basal-, mid- and apical-levels of the ventricle. Sections were stained with DAB and counterstained with Gill’s haemotoxylin. * P < 0.05 vs. control; # P < 0.05 vs. saline-treated group. Each data point represents mean ± SEM. (n = 4 animals per group).
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Figure 3.10: Collagen I immunohistochemistry of cardiac sections. Figures A – F show representative digital images of collagen I staining: (A) negative control; (B) naïve control; (C) saline-treated; (D) diazepam (DZP)-treated; (E) atenolol-treated; and (F) DZP + atenolol-treated seizure animals. Figures A – F were captured at 40 x magnification. Scale bars represent 100 µm. Graphs G - I demonstrate quantification (% collagen volume fraction; CVF) of collagen I staining in each transverse ventricle sections at the basal-, mid- and apical-levels of the ventricle. Sections were stained with DAB and counterstained with Gill’s haematoxylin. * P < 0.05 vs. control; # P < 0.05 vs. saline-treated group. Each data point represents mean ± SEM. (n = 4 animals per group).
Figure 3.11: $\alpha$-smooth muscle actin ($\alpha$SMA) immunohistochemistry of cardiac sections. Figure A demonstrates negative control with lack of blood vessel staining, taken at 40 x magnification. Figure B is an image showing $\alpha$SMA-stained cardiac sections of a 7 day diazepam + atenolol-treated animal; the arrowhead shows a positively stained blood vessel, and the arrow shows light diffuse staining of myofibroblasts, co-localised with an infarct, which also highly stains with collagen I (Figure C, arrow). Figures B – C were captured at 20 x magnification. Scale bars represent 100 $\mu$m. Graphs D - F demonstrate quantification of $\alpha$SMA staining in each transverse ventricle sections at the basal-, mid- and apical-levels of the ventricle. Sections were stained with DAB and counterstained with Gill’s haemotoxylin. No results were found to be significant compared to control and saline-treated animals. ($n = 4$ animals per group). Each data point represents mean ± SEM.
Chapter 4: Discussion
This study demonstrated clear detrimental ECG changes and cardiac injury following KA-induced seizures in rats. These changes are mediated through a central sympathetic surge, subsequent tachycardia, and pathological remodelling. Unexpectedly, attenuating the seizures with DZP alone failed to prevent the ECG changes or myocardial damage. Atenolol intervention, alone or in combination with DZP, was cardioprotective against seizure-induced cardiac injury. The mechanisms by which these cardiac changes arise will be discussed.

4.1 Behavioural Changes

Intrahippocampal KA administration rapidly induced high-level seizure behaviours and these often progressed to generalised seizures (Levels 4 – 5 behaviours). Raedt et al. (2009) also showed a similar dose of intrahippocampal KA (1.14 nmol) induced motor seizures within 66 ± 15 minutes, with sustained SE for over 7 hours, and seizure activity present up to 24 hours. We also observed increased behavioural seizure scores at 24 hours post-KA in the saline- and atenolol-treated groups.

In this study, atenolol intervention alone exerted no significant reduction in seizure behaviours, and this lack of anticonvulsant property has been observed by others, even up to a dose 50 mg/kg (ip.) (Damasceno et al., 2013; De Sarro et al., 2002; Little & Bealer, 2012; Luchowska et al., 2002). Some lipophilic β-blockers have been reported to exert anti-convulsant properties, attributable to central β-adrenergic blockade (Fischer, 2002; Nakamura et al., 2008; Raju et al., 1998). Our group has recently demonstrated that disruption of the BBB occurs at 3 hours following intrahippocampal KA-induced seizures (unpublished data), suggesting that atenolol has the potential to penetrate the BBB following seizures and exert anticonvulsant effects. Analysis conducted in these rats as part of a separate study (Read, 2014) did show a significant atenolol-associated decrease in seizure EEG activity, suggesting that atenolol treatment, especially in a prophylactic setting, may provide neurological protection.

DZP is reported to be a very effective anticonvulsant in animal seizure models (Baran & Mevissen, 1994; Morrisett et al., 1987; Pollard et al., 1994). The observed motor defects (e.g. ataxia) associated with DZP intervention have also been seen by others (Baran & Mevissen, 1994; Luchowska et al., 2002). Jones et al. (2002) reported the ED\textsubscript{50} of DZP in a Li-Pilo seizure model increased from 4.8 mg/kg (ip.) at 10 minutes post-SE, to 100 mg/kg at 45 minutes, attributing this to GABA\textsubscript{A} receptor desensitisation. While pilocarpine-induced seizures are reported to be more severe than KA-induced seizures (Curia et al., 2008), we found that a 5 mg/kg dose (sc.) of DZP delivered at 60 minutes post-KA effectively
attenuated seizures in all our animals. Interestingly, the lipophilic β-blockers, metoprolol and propranolol, but not atenolol, have been shown to enhance the anticonvulsant effect of DZP in electrically-induced seizures, indicating these synergistic effects are probably related to central adrenergic blockade exerting additional anticonvulsant effects (De Sarro et al., 2002; Luchowska et al., 2002).

4.1.1 Body Weights
Seizures have previously been reported to cause weight loss in a seizure animal model (Damasceno et al., 2013). We found saline-treated rats lost significant body weight in the first 24 hours, however, no other intervention groups demonstrated this acute weight loss. As isoprenaline administration is reported to have no effect on body weights (Shizukuda et al., 1998), the weight loss is unlikely to be directly related to sympathetic stimulation, and is more likely attributable to a combination of high intensity motor seizures and central autonomic dysfunction affecting eating and drinking abilities. As only saline-treated seizure rats demonstrated a significant reduction below their respective baseline, this suggests that all other interventions were beneficial in some respect; DZP is reported to induce hyperphagia (Naruse et al., 1991; Naruse et al., 1988; Naruse & Ishii, 1995), although the attenuation of seizures is mostly likely accountable for animals losing less weight. The observed improvement in body weights in combination-treated animals at days 13 – 14, suggests that this treatment may be providing beneficial effects on overall animal well-being. The lower weights observed in the saline group at 13 – 14 days post-KA may be due to related to chronic autonomic dysfunction, as has been reported in in hypertensive rats (Ikeda et al., 1999).

4.2 Cardiovascular Changes

4.2.1 Heart rate
To our knowledge, there have been no previous studies evaluating cardiac changes in the conscious intrahippocampal KA-induced seizure model. We showed KA administration (2 nmol) into the dorsal hippocampus resulted in a rapid increase in HR, coincided with high-level seizure behaviours. Seizurogenic discharges originating in the hippocampus may spread to other nearby sympathetic nuclei, producing cardiovascular effects (Ross et al., 1984; Talman et al., 1981). Other seizure models have also demonstrated tachycardia following seizure induction (Bealer et al., 2010; Metcalf et al., 2009a). HR’s of saline-treated animals in our study, as well as other studies, showed a return to baseline by 24 hours, suggesting
predominant HR changes occur in the initial seizure period when there is widespread autonomic dysfunction (Bealer & Little, 2013; Little & Bealer, 2012; Powell et al., 2014). Interestingly, no bradycardia was observed in our seizure model; systemic KA administration induces bradycardic followed by tachycardic periods, suggesting systemic KA may exert central effects on parasympathetic nuclei or peripheral effects on the vagal nerve (Read et al., 2014; Sakamoto et al., 2008). The presence of premature atrial contractions were also noted in this study in some animals (Figure 3.4B), suggesting increased risk of arrhythmias, and this has been observed during seizures in patients (Bateman et al., 2008).

4.2.1.1 Diazepam-mediated effects

Disappointingly, DZP intervention did not attenuate the tachycardia, even though seizures were greatly reduced. Due to this study lacking a sham-DZP control group, we are unable to elucidate whether this tachycardia is due to remnant seizure activity within central autonomic areas inducing an increase in HR, or whether DZP itself exerts cardiostimulatory effects. Previously, we found that an acute high dose of DZP (10 mg/kg, sc.) in naïve rats induced significant tachycardia (Millen et al., 2013), suggesting DZP exerts direct or indirect cardiostimulatory effects. Additionally, as we observed higher HR’s in the DZP-treated group compared to the saline-treated group at both 24 and 48 hours, after seizure cessation, this further suggests that DZP itself is inducing cardiostimulatory effects. DZP is reported to induce increases in HR through mediation of two central autonomic nuclei. Firstly, microinjection of DZP into the NTS potentiated the effects of isoguvacine (GABA<sub>A</sub> receptor agonist)-induced HR and BP increases, via disinhibition of sympathetic outflow (Barron et al., 1997). Secondly, DZP modulation of GABAergic neurons in the nucleus ambiguus would cause direct vagal inhibition, also potentially leading to increased HR and BP (Bentzen & Grunnet, 2011).

DZP may also exert direct effects on cardiomyocytes, leading to increased HR. DZP is reported to potentiate the inotropic effects of catecholamines in vitro (Juan-Fita et al., 2003; Marin & Hernandez, 2002; Martinez et al., 1995). Interestingly, the observation of increased HR’s in DZP animals above saline animals at 24 and 48 hours coincides with the high levels of NA in these animals as analysed elsewhere (Read, 2014). This suggests that DZP is indeed potentiating the adrenergic effects of catecholamines. Carmen Collado et al. (1998) demonstrated in in vitro ventricular myocardium strip studies that DZP inhibits the cardiac enzyme phosphodiesterase (PDE) 4. PDE is the sole enzyme responsible for the breakdown of cAMP, and is therefore crucial in maintaining cAMP homeostasis (Zaccolo, 2006). Accumulation of cAMP within cardiomyocytes has several downstream effects: (1) increased
PKA will increase downstream modulation of calcium handling (via LTCC, RyR etc.) leading to increased inotropic and chronotropic responses (Zaccolo, 2006); (2) loss of cAMP compartmentalisation may exaggerate cardiac inotropic and chronotropic responses (Zaccolo, 2006); and (3) activation of cAMP-gated HCN channels within pacemaker cells will increase cardiac rate (Landry & Gies, 2008). Interestingly, Powell et al. (2014) found evidence of decreased cardiac HCN2 mRNA in rats 9 weeks following SE induction by KA that were incidentally attenuated by DZP (4 mg/kg, ip.); however, it is unclear if this down-regulation is associated with an acute DZP dose or is due to long-term seizure effects. PDE4 inhibition is also thought to prevent the β2 G-protein switch from Gs to Gi, thereby preventing the cardioprotective effects of β2-Gi coupling; this mechanism involves β-arrestin (Baillie & Houslay, 2005; Lynch et al., 2005; Xiao, 2001). Indeed, DZP has been demonstrated to cause alterations in Ca2+ handling in vitro in ventricular cells, however it is unclear whether these alterations are associated with cAMP dysregulation or by direct channel modulation (Akahane et al., 1987; Earl & Tietz, 2011; Hernández, 1991; Kanaya et al., 2002; Kanaya et al., 2006; Nonaka et al., 1997; Yamakage et al., 1999). Additionally, DZP may also exert cardiac effects through binding to peripheral benzodiazepine sites. GABA\A receptors are proposed to be located on sinus node, and DZP may modulate these receptors to effect sinus node rhythm (Matsuyama et al., 1993). Benzodiazepines, including DZP, are also known to bind to the translocator protein (TSPO), which is located in the outer mitochondrial membrane within various tissues including cardiac ventricles, adrenal tissue, kidneys, brain, smooth muscle cells, and monocytes (Davies & Huston, 1981; Gehlert et al., 1985; Papadopoulos et al., 2006; Veenman et al., 2008). TSPO is involved in mitochondrial respiration, inflammation, and cell proliferation, and is also implicated in apoptosis and mitochondrial permeability transition pore (MPTP) opening (Rupprecht et al., 2010). There are mixed results on the inotropic effects of TSPO activation in the heart, although TSPO activation appears to induce detrimental cardiac effects, including exaggeration of ischaemic-reperfusion injury, and pro-arrhythmic effects (Surinkaew et al., 2011). As DZP is a ligand at TSPO, there is a possibility that DZP may induce cardiac damage through a TSPO-dependent effect (Veenman et al., 2007). Interestingly, in a seizure model, TSPO expression was increased in the ventricles 30 minutes after electrically-induced seizures, potentially implicating TSPO in apoptosis or necrosis (Basile et al., 1987). While not directly accounting for the observed increased HR following DZP treatment, DZP-mediated effects via the TSPO may account for the incurred cardiac injury.
Figure 4.1: Central and direct cardiac effects of diazepam (DZP) on heart rate and myocardial cell death. nAmb: nucleus ambiguus; NTS: nucleus tractus solitarii; GABAAR: γ-aminobutyric acid-A receptor; TSPO: translocator protein; PDE4: phosphodiesterase 4; cAMP: cyclic adenosine monophosphate; HCN: hyperpolarization-activated cyclic nucleotide-gated channel; PKA: protein kinase A; [Ca\(^{2+}\)]: intracellular Ca\(^{2+}\); β2: β2 adrenergic receptor, coupled to Gs or Gi protein.

4.2.1.2 Atenolol Treatment

Atenolol intervention, both alone and in combination with DZP, rapidly decreased HR to baseline levels, and these HR’s remained at baseline through the 7-day study. Bealer et al. (2010) found atenolol pretreatment of Li-Pilo seizures prevent the significant tachycardia observed at 90 minutes post-SE induction. In agreement with other research publications, atenolol treatment did not reduce HR significantly below baseline (Bealer et al., 2010; Little & Bealer, 2012). This lack of attenuation of HR below baseline may consequently be due to the chronotropic effect of sympathetic stimulation and catecholamine release on β2 receptors.

Although DZP has been shown to induce positive chronotropic and inotropic responses, in our study, combination of DZP + atenolol did not demonstrate any signs of DZP-induced tachycardia, although there was one time point (t = 90 minutes post-KA) where combination-treated animal HR’s were increased significantly above atenolol-treated animal HR’s. Interestingly, selective β1 blockade or non-selective β blockade, but not selective β2 blockade, prevents the positive inotropic responses of DZP \textit{in vitro} (Akahane et al., 1987; Conahan & Vogel, 1986; Marin & Hernandez, 2002). This suggests that β1 antagonism with atenolol prevents the excessive β1-induced cAMP synthesis, and therefore the downstream effects of DZP-induced PDE4 inhibition has little consequence. However, practolol treatment (β1 antagonist, 2.5 mg/kg; iv.) was reported to have no effect on DZP-induced tachycardia \textit{in vivo} (10 mg/kg, po.), which is not in accordance with our results (Gerold et al., 1976). This suggests that DZP may also exert chronotropic cardiac effects independent of the β1 receptor, by central sympathetic stimulation, or by TSPO or Ca\(^{2+}\) channel modulation (Figure 4.1).
4.2.2 QT intervals

The tachycardia observed within the first 60 minutes of KA administration coincided with significant QTc prolongation, and this has also been demonstrated in our laboratory following systemic KA administration (Read et al., 2014). Importantly, QTc intervals remained prolonged in saline-treated animals throughout the 7 day study, and other seizure models have also demonstrated prolonged QTc intervals, up to 9 weeks post seizure-induction (Bealer & Little, 2013; Metcalf et al., 2009a; Powell et al., 2014). Prolongation of the QT interval is significant as it increases the risk of fatal arrhythmias occurring such as torsades de pointes.

Clinically, QT prolongation is observed during seizures (Brotherstone et al., 2010; Seyal et al., 2011; Tavernor et al., 1996). QT prolongation leading to fatal arrhythmias may be implicated in the pathogenesis of SUDEP (Feldman et al., 2013). Conversely, arrhythmias and syncope can also present with involuntary movements resembling that of a seizure, leading to the possibility of misdiagnosing epilepsy and SUDEP. Therefore, both EEG and ECG data are important to correctly distinguish between cardiogenic syncope leading to sudden death, and SUDEP (Bergfeldt, 2003).

Many causes of QT prolongation may be implicated in our study, for example, cerebral dysfunction, myocardial ischaemia, release of stress hormones, hypoxia, and various channelopathies (Feldman & Gidal, 2013; Surges et al., 2010). Our observation of decreased SpO₂ also implies the occurrence of hypoxia, which can activate specific K⁺ channels leading to repolarisation abnormalities (Hool, 2005). High levels of catecholamines may also prolong the QT interval via sympathetically-induced increases in calcium influx (Surges et al., 2010). Various channelopathies are reported in both epileptics and in animal models, which may contribute to QT prolongation. In particular, mutations in various voltage-gated potassium and sodium channels have been demonstrated in SUDEP cases (Feldman & Gidal, 2013; Tu et al., 2011a; Tu et al., 2011b). Additionally, HCN and voltage-gated K⁺ channels (Kv4.2) have both been found to be down-regulated in seizure models, and this can also contribute to QT prolongation (Bealer et al., 2010; Ludwig et al., 2003; Powell et al., 2014).

DZP-treated animals demonstrated significantly prolonged QTc intervals up to 48 hours post-KA, and this prolongation has also been observed in monkeys treated with DZP (Moscardo et al., 2010). QTc prolongation in these animals may be precipitated by the above mechanisms similar to those in saline animals. Atenolol treatment, both alone and in combination, rapidly reduced QTc intervals to baseline levels, and these remained reduced through the 7 day study. Others have also reported atenolol to effectively reduce QTc prolongation in seizure models (Bealer et al., 2010; Damasceno et al., 2013). Little and Bealer (2012) found that pretreatment
with a single dose of atenolol did not reduce QTc interval to control levels at 24 hours post-SE, implying that regular treatment with atenolol is necessary to provide adequate cardioprotection against arrhythmias.

### 4.2.3 T wave amplitude

This study found no significant changes in T wave amplitude, apart from DZP-treated animals at 7 days post-KA. However, this study was carried out as part of a larger study, and a significant increase in T wave amplitude was seen with higher \( n \) numbers (Read, 2014). Furthermore, T wave elevation has also been observed following systemic KA (Read et al., 2014; Read, 2014). The decrease in T wave amplitude of DZP-treated animals at 7 days were likely caused by ischaemia, as this is reported to cause accumulation of extracellular K\(^+\) leading to a reduction in T wave amplitude (Antzelevitch, 2001). Pentobarbital is reported to reduce T wave amplitude by decreasing the transmural dispersion of repolarisation in the ventricle, and the similar mechanisms of actions of pentobarbital and DZP (i.e. GABA\(_A\) receptor positive allosteric modulators) suggest this may be involved (Antzelevitch, 2001; Shimizu et al., 1999).

### 4.2.4 Blood pressures

Several animal studies demonstrate increased BP’s, both acutely and at 24 hours post-seizure induction (Bealer et al., 2010; Little & Bealer, 2012; Metcalf et al., 2009a). In our study, BP’s were only increased in the DZP group at 1 hour post-KA. This is likely attributable to having very small \( n \) numbers (\( n = 2 – 4 \)). We had expected all animals to exhibit increased BP’s within the initial 3 hour period post-KA, and indeed, our laboratory did find this when \( n \) numbers were higher (Read, 2014). It should be noted however, that the two rats in the DZP group exhibited very high-level seizures, potentially accounting for the largely increased BP’s. BP is regulated by vascular resistance, HR, myocardial contractility and end-diastolic volume, therefore, sympathetic stimulation of adrenergic receptors increases BP (Guyenet, 2006). Sympathetically-mediated activation of the renin-angiotensin-system also exerts powerful vasoconstriction (Lavoie & Sigmund, 2003). Elevations in BP can contribute to increased cardiac afterload, potentiating cardiac dysfunction and injury, in addition to increasing risk of MI and stroke (Mayet & Hughes, 2003; Vaughan & Delanty, 2000). Seizure discharges within central pressor regions (e.g. NTS) may explain the initial increases in BP (i.e. within 3 hours), however increased BP’s at prolonged time points suggest chronic neurohormonal or cardiovascular changes (Thomas, 2011). Bealer et al. (2010) reported atenolol pretreatment had no effect on BP in rats at 90 minutes post-SE induction, which is
likely attributable to $\alpha_1$ adrenergic receptors primarily producing peripheral vasoconstriction (Thomas, 2011). This suggests that carvedilol, a non-selective $\beta$-blocker with $\alpha_1$ antagonistic properties may be effective in reducing both cardiac and vascular changes consequent to seizures (Gentles, 2009). DZP is often associated with hypotension, in both humans and animals, which is considered to be via central cardiodepressant actions (Delaunois et al., 2009; Mehta et al., 2007; Sigg & Sigg, 1969). However, we and others have observed the opposite, with increased BP’s seen at 3 hours post-KA in DZP-treated animals (Mailliet et al., 2001). This may either be related to the DZP-induced tachycardia, or may be via central pressor modulation, as previously described (refer to Chapter 4.2.1.1). However, due to the lack of statistical power, we cannot conclude much from these BP results and further investigation is required.

4.2.5 SpO$_2$

Oxygen desaturation below 70% is commonly reported following both partial and generalised seizures in patients, and are particularly noted in seizures with right temporal onset (Bateman et al., 2008; Seyal et al., 2010; Seyal et al., 2011). To our knowledge, there are no known reports on SpO$_2$ values in animal seizure models. As partial seizures are also associated with apnoea and decreased SpO$_2$, this implies that a central mechanism, in contrast to increased metabolic demand due to motor seizures, is due to the observed SpO$_2$ falls (Seyal et al., 2010). Furthermore, oxygen desaturation is often accompanied by increased end tidal carbon dioxide levels signifying hypoventilation is involved in decreased SpO$_2$ values (Bateman et al., 2008). In our study, a rat that died at 90 minutes post-KA (atenolol treatment) developed severe SE and exhibited respiratory difficulties prior to death, with a SpO$_2$ of 85.2% at 60 minutes post-KA; these changes may be via a central hypoventilation mechanism, or potentially neurogenic pulmonary oedema, as is often seen in SUDEP cases (Devinsky, 2004).

It is not clear why we observed decreased SpO$_2$ in DZP- and atenolol-treated animals, but not in saline and combination-treated animals. An acute reduction in SpO$_2$ in saline-treated animals was expected as a result of high-level motor seizures combined with central respiratory dysfunction, however this was not seen, even though animals did often exhibit changes in respiration patterns (e.g. heavy or rapid breathing). The reduction in SpO$_2$ of DZP-treated animals may have been via central respiratory depression, with the presence of tachycardia (and increased cardiac oxygen demand) potentiating this reduction (Chapman et al., 2001). It was not expected that atenolol would reduce SpO$_2$. However, this reduction may be due to high-level motor seizures causing increased peripheral oxygen demand, in
combination with central respiratory dysfunction and reduced cardiac output, not sufficient to maintain appropriate oxygenation levels. Previous studies have shown that atenolol administration during exercise reduces both cardiac output and maximal oxygen consumption, suggesting an imbalance in oxygen delivery and supply, resulting in decreased SpO₂ levels during seizures (Bouckaert et al., 1989; Svendsen et al., 1979; Van Bortel & van Baak, 1992). The lack of reduction in SpO₂ in the combination group may be related to the attenuation of seizures preventing the central respiratory dysfunction and increased peripheral oxygen demand, combined with atenolol-treatment preventing the tachycardia and cardiac dysfunction. However, further work is required in this area to clarify these results.

4.3 **Structural Changes**

Histological and immunohistochemical analysis of our hearts showed cardiac injury, evidenced by inflammatory cell infiltration, cell death, fibrosis, and oedema, indicating the occurrence of cardiac remodelling. The mechanisms by which cardiac injury occurs in our seizure model is likely due to several factors that will be discussed, involving tachycardia-induced ischaemia, direct catecholamine-induced cardiotoxicity, and subsequent ventricular remodelling.

4.3.1 **Oedema**

Acute ischaemic cardiac injury is associated with myocardial interstitial oedema, relating to contractile dysfunction causing alterations in microvascular permeability and reductions in lymph flow (Friedrich, 2010). Interstitial oedema has previously been reported in isoprenaline-treated rats, in post-mortem SUDEP cases, and in rats following seizure induction (Bertinchant et al., 2000; Read et al., 2014; Thom, 2007). Although myocardial oedema is considered a reversible form of ischaemic injury, the presence of oedema in 14 day saline-treated animals suggests continual cardiac dysfunction impairing the retraction of this interstitial fluid (Friedrich, 2010). This may explain the cause of myocardial oedema in saline- and DZP-treated animals, and indeed, echocardiography carried out elsewhere on these animals did demonstrate systolic dysfunction (Read, 2014). The presence of myocardial oedema is also important as it induces additional contractile dysfunction and increases the risk of arrhythmias (Friedrich, 2010). Furthermore, chronic myocardial oedema can also induce interstitial fibrosis, which is thought to involve changes in intramyocardial pressures and the extravasation of cellular proteins (Desai et al., 2008; Friedrich, 2010). This suggests that collagen I deposition in saline-treated animals is partly attributable to the observed interstitial oedema in these animals. β₁-blockade with esmolol has previously been reported to reduce
myocardial oedema in animals undergoing cardiac support, by maintaining myocardial lymph and blood flows, as well as reducing myocardial oxygen demand and ischaemia (Mehlhorn et al., 1996). Similar mechanisms are likely related to the observed reductions in oedema in atenolol and combination groups, and this may further be attributable to the reductions in interstitial fibrosis in these groups.

4.3.2 Inflammatory response

Infiltration of myocardial CD68-positive cells has been shown in multiple animal models including isoprenaline, MI, and renal hypertension studies (Nakatsuji et al., 1997; Nicoletti et al., 1996; Shizukuda et al., 1998; Tan et al., 2010). In the isoprenaline studies, macrophage infiltration was evident within 12 hours of isoprenaline administration, with elevated levels of CD68+ cells still seen at 7 – 14 days (Nakatsuji et al., 1997; Shizukuda et al., 1998). Furthermore, infiltration of macrophagic monocytes have been demonstrated within 8 hours following NA administration to rats, becoming widespread by 24 hours (Neri et al., 2007).

We observed similar inflammatory responses to these catecholamine studies, and this may be associated with increased sympathetic output following seizure. Indeed, the sympathetic system is involved in activating the early phase reaction of inflammation (Black, 2002). Furthermore, NA and adrenaline enhance pro-inflammatory cytokine release from macrophages, via a NFκB (nuclear factor κ-light-chain-enhancer of activated B cells) mechanism (Flierl et al., 2009). Atenolol-mediated attenuation of the inflammatory response is not likely via a direct effect on leukocytes, as these cells are reported to express mostly β2 receptors (Barnes, 1999). Rather, it is likely that attenuation of the tachycardia, and subsequent ischaemia, prevented release of pro-inflammatory cytokines. Of note, the rat that died 1.5 hours post-KA (atenolol-treated) exhibited intense subendocardial CD68+ cell infiltration (Figure 3.9H); macrophage infiltration has been seen this early following cardiac ischaemia, and therefore suggests this rat may have had an MI (Kakio et al., 2000). The high expression of CD68+ cells in DZP-treated rat hearts may be related to sympathetically-induced inflammatory responses, as described for saline rats. One study has also reported myocardial mononuclear cell infiltration following chronic DZP administration (1 mg/kg, po., for 4 weeks) in rats, but the authors did not examine whether this was related to HR changes (Mousa, 2014). Interestingly, DZP (10 mg/kg, ip.) has previously been shown to reduce acute inflammatory response in rats, which the authors contributed to the anti-inflammatory effects of the TSPO (Lazzarini et al., 2001). However, in this study, the beneficial TSPO effects are probably overcome by high-level catecholamines from seizures inducing cardiotoxic and pro-inflammatory responses.
4.3.3 Cell death

Levels of apoptosis are very low in the healthy heart, approximately 0.001 – 0.01\% (Whelan et al., 2010). Although apoptosis rates in HF also remain very low (0.08 – 0.25\%), this can accumulate over time due to the limited ability of cardiomyocytes to proliferate, with possible progression to a cardiomyopathy (Mani & Kitsis, 2003; Whelan et al., 2010). Numerous studies with adrenergic agonists have demonstrated induction of significant cardiomyocyte apoptosis (Goldspink et al., 2004; Krishnamurthy et al., 2007; Neri et al., 2007; Shizukuda et al., 1998). Goldspink et al. (2004) found that isoprenaline administration in rats induced the highest rate of cell death (both necrosis and apoptosis) at 2.2 mm from the apex, corresponding to our apical-ventricle level sections. However, we cannot compare our apoptosis values between ventricle levels as they were calculated as a raw value and did not take into account the difference in cross-sectional areas between levels. *In vitro* studies also demonstrate ventricular myocyte pacing induces apoptosis, involving increased [Ca$^{2+}$], leading to activation of the mitochondrial apoptotic pathway (Aoki et al., 2002; Kuramochi et al., 2006; Remondino et al., 2003; Sepúlveda et al., 2013). Catecholamines can directly induce cardiomyocyte apoptosis by $\beta_1$-dependent mechanisms involving adenylyl cyclase-cAMP, Ca$^{2+}$/calmodulin kinase II (CaMKII), and mitogen-activated protein kinase (MAPK) signalling pathways (Figure 4.2) (Yang et al., 2013; Zhu et al., 2003). $\beta$-adrenergic agonists acting on $G_s$-coupled receptors to elevate cAMP levels are reported to increase myocardial oxygen demand and also redistribute blood flow away from the subendocardium, rendering it ischaemic, and at risk of undergoing apoptosis (Leineweber et al., 2006). Catecholamines may also induce oxidative damage independent of receptor activation, via auto-oxidation into reactive oxygen species intermediates (Neri et al., 2007). As the apoptotic process is thought to take hours to days, the presence of apoptosis at days 7 and 14 in saline-treated animals suggests the presence of a chronic pro-apoptotic stimulus (Green, 2005). This is not likely related to tachycardia or NA levels, as these were both normalised at 7 and 14 days, therefore the apoptosis may instead be induced by ischaemia caused by reported systolic dysfunction in these animals (Read, 2014). Previously, in both MI and isoprenaline animal studies, apoptosis was found to peak at 3 – 6 hours following the initial stimulus (Goldspink et al., 2004; Kajstura et al., 1996). This suggests we may have found higher apoptosis rates had we examined the cardiac tissue within 24 hours following seizure induction when tachycardia was most prevalent.

$\beta_1$ blockade has been found to inhibit apoptosis *in vitro* and *in vivo* following catecholamine administration, which are in accordance with the protective effect of atenolol against
apo
2ptosis seen in this study (Goldspink et al., 2004; Zaugg et al., 2000). Additionally, it is possible that chronically high catecholamines may have induced a switch of the $G_s$-coupled $\beta_2$ receptor to $G_i$, therefore causing downstream anti-apoptotic effects via phosphoinositide 3-kinase (PI3K)-Akt (protein kinase B) signalling (Chesley et al., 2000; Xiao, 2001). In this case, atenolol treatment would prevent the pathological $\beta_1$ pro-apoptotic signalling, sparing the protective $\beta_2$ anti-apoptotic signalling, however, adrenergic receptor expression was not examined in this study. To our knowledge, the effects of DZP on cardiac apoptosis have not previously been investigated. DZP intervention exerted no anti-apoptotic effects in rat hearts, and similarly to saline-treated animals, this may be due to tachycardia-induced ischaemia and calcium overloading, or $\beta_1$ pro-apoptotic mechanisms (Yang et al., 2013; Zhu et al., 2003). Additionally, the TSPO is comprised of the adenine nucleotide translocator and voltage-dependent anion channel units within the mitochondrial membrane, both of which are involved in MPTP formation (Kinnally et al., 2011); as MPTP is implicated in apoptosis and necrosis pathways, there is a possibility for TSPO to be involved in cell death, however a recent study suggested TSPO was not involved in MPTP regulation (Šileikytė et al., 2014).

As heart sections were not co-stained with a cardiomyocyte antibody in this study, we cannot confirm the labelled apoptotic cells are in fact cardiomyocytes. Indeed, some apoptotic cells appeared spindle-shaped like fibroblasts, with other apoptotic cells highly localised in areas also showing high CD68$^+$ staining, suggesting they were apoptotic inflammatory cells.
Correlation analysis between apoptotic and CD68\(^+\) cell numbers showed an \(r^2\) value of 0.59 (data not shown), suggesting a low-level of correlation; this indicates either (1) the macrophages are undergoing of apoptosis; or (2) hearts that have more macrophage infiltration are more damaged and have increased numbers of apoptotic cells (comprising cardiomyocytes, fibroblasts, and inflammatory cells). Ikeda et al. (1999) demonstrated that TUNEL-positive cells in a hypertensive rat model were exclusively non-cardiomyocytes. Additionally, cardiomyocytes express low endogenous levels of certain pro-apoptotic proteins (Apafl), suggesting they are at decreased risk of apoptosis induction, and non-myocyte cells are at increased risk; this may account for our high apoptosis levels, possibly in non-myocyte cells (Chiong et al., 2011).

### 4.3.4 Troponins

Measurements of circulating troponins are a clinical diagnostic tool for assessing cardiac damage. Although considered to be released exclusively from necrotic cells, troponins can in fact also be released from cardiomyocytes following apoptosis, myocyte turnover, increased cell wall permeability, membrane blebbing, and release of troponin degradation products (reviewed by White, 2011). However, due to a small amount (5 – 8%) of total troponins being located unbound in the cytoplasm, reversible cardiomyocyte injury may also elicit small fluctuations in circulating troponin levels (White, 2011). Rapid atrial pacing in humans was found to increase cardiac troponin T levels, implying that tachycardia alone can increase troponin release, potentially through ischaemia, a stretch-mediated mechanism, or via low level cardiomyocyte necrosis (Turer et al., 2011). Isoprenaline is also reported to increase cTnI levels in rats (Bertinchant et al., 2000; Clements et al., 2010). Taking the above into account, increased cTnI levels in our study are likely due to a combination of ischaemic damage and necrotic and apoptotic cardiomyocytes. Bertinchant et al. (2000) found that a rise in cTnI to at least 0.35 ng/ml was required for observable histological damage to occur. Troponin levels in this study only reached a maximum of 0.229 ± 0.03 ng/ml, yet we still observed histological evidence of micro-infarcts, oedema and fibrosis.

Clinically, there are previous reports of no change in troponin levels following seizures (Adjei, 2011; Mehrpour et al., 2013; Woodruff et al., 2003), however Hajsadeghi et al. (2009) reported increased cTnI levels in patients exhibiting more than three recurrent seizures. Additionally, Soundarya et al. (2014) conducted a meta-analysis on troponin levels in SE, and found a 4 – 5 fold increase in cTnI levels in patients already exhibiting coronary artery disease, and this further correlated with an increased 30-day mortality.
Chapter 4: Discussion

We observed significantly increased cTnI levels at 24 hours in saline- and DZP-treated animals. Others have also reported increased cTnI to approximately 0.3 – 0.7 ng/ml at 60 minutes post-SE induction with pilocarpine (Bealer et al., 2010; Metcalf et al., 2009a). Our values may have been this high had we measured cTnI earlier, however we believed it was too stressful for the rats to take blood samples whilst they were still experiencing high-level seizures. Atenolol pre-treatment is reported to prevent increased cTnI at 60 minutes post-SE induction (Bealer et al., 2010). Likewise in our study, atenolol- and combination-treated animals did not exhibit any increases in cTnI levels at 24 hours, likely attributable to preventing the excessive tachycardia and β1-mediated cell death that was seen in saline- and DZP-treated animals. In contrast, the increased cTnI levels in DZP-treated animals at 24 hours may be attributable to the prolonged tachycardia and possible ischaemic damage, or by direct DZP cardiotoxic effects, as described in Chapter 4.2.1.1.

4.3.5 Fibrosis

Our values for collagen I expression, both in naïve and seizure animals, were much higher than reported in literature, and this is probably related to overexposure of substrate visualisation during the staining protocol. However, our observed ~1.5-fold increases in collagen I are still comparable to relative increases seen in other studies (Diez et al., 1996; Varo et al., 1999; Weber et al., 1988). NA infusion was previously reported to significantly increase collagen I mRNA after 4 days, with a mild elevation still present at 14 days (Briest et al., 2001); this suggests that collagen remodelling at a transcriptional level occurs within the first 3 – 4 days, which may support our results demonstrating no further collagen deposition at 14 days compared to 7 days. Increased cardiac fibroblasts have recently been reported following chronic DZP treatment in rats (Mousa, 2014). In our study, hearts from DZP-treated animals demonstrated high collagen I deposition, and this is probably as a result of DZP-induced ischaemic damage.

Cardiac fibroblasts express the β2 receptor, with adrenergic agonists reported to increase cardiac fibroblast proliferation, although there are mixed reports on myofibroblast differentiation (Nakatsuji et al., 1997; Porter & Turner, 2009; Turner et al., 2003). Non-selective and β2-selective β-blockers are reported to inhibit cardiac fibroblast proliferation and to also inhibit release of pro-inflammatory cytokines and growth factors (reviewed by Porter & Turner, 2009). This suggests that carvedilol may be useful in preventing fibrosis. Furthermore, it is likely that the reduction in collagen I in our study with atenolol-treatments were due to inhibition of cardiomyocyte β1 signalling reducing inotropy, chronotropy, and
subsequent remodelling responses. However, atenolol has been reported to reduce collagen I deposition in hamsters with HF (Mansoor et al., 1996). Atenolol treatment would also have the additional benefit of preventing renin-angiotensin-system activation by β₁ receptors, of which angiotensin II exerts pro-fibrotic effects by the angiotensin type I receptor (Mehta & Griendling, 2007). As there were no major structural changes observed in atenolol- and combination-treated rat hearts, it appears that the seizure-associated cardiac changes that occurred in the initial 60 minutes following seizure induction, are not prolonged enough to elicit remodelling responses.

The presence of cardiac myofibroblasts is indicative of cardiac injury, as these cells are not present in the healthy myocardium. Myofibroblasts have been observed following isoprenaline, pressure overload, and MI (Cleutjens et al., 1995; Leslie et al., 1991; Nakatsuji et al., 1997). Interestingly, in humans, αSMA positive-cells have been located within post-infarct scars, but not within interstitial fibrosis, suggesting these remodelling processes arise from different hormonal, haemodynamic, mechanical and inflammatory mechanisms (Suurmeijer et al., 2003).

The lack of αSMA expression in our study outside of blood vessels, even in areas of interstitial fibrosis, suggest that a ‘threshold’ stimulus for myofibroblast phenotype transition was not reached. This absence of αSMA-positive cells within interstitial fibrosis has also been seen in humans, with myofibroblasts only present within post-infarct scars (Suurmeijer et al., 2003). Indeed, we found an infarcted area with concurrent high expression of both αSMA and collagen I, in a 7-day combination-treated rat (Figures 3.11B-C). Interestingly, this rat was found to have a HR of 588 b.p.m. during the initial 60 minute seizure period, suggesting that significant irreversible ischaemic cardiac injury may have occurred during this time. Furthermore, combination intervention was not able to prevent the subsequent remodelling process. This reinforces that prophylactic cardioprotective treatment should be indicated in patients prone to seizures.

4.4 Relevance of Pathology to Known Cardiomyopathies

Several features of the cardiac changes observed in this study are similar to other specific cardiomyopathies: tachycardia-induced cardiomyopathy (TIC) and stress cardiomyopathy (Takotsubo syndrome; SCM). Relating to these cardiac disorders may help elucidate the mechanisms involved in seizure-induced cardiomyopathy, and how best to manage it.
4.4.1 Tachycardia-induced cardiomyopathy

TIC is described as a reversible cardiac condition in which prolonged tachycardia leads to myocardial dysfunction; in particular, TIC may progress to left ventricular dysfunction, HF, or even sudden death (Ellis & Josephson, 2013; Khasnis et al., 2005; Nerheim et al., 2004; Pak et al., 1997). Mechanisms involved in the pathogenesis of TIC are thought to involve alterations in myocardial energy use, abnormal calcium handling, myocardial ischaemia, and extensive remodelling (Lishmanov et al., 2010; Shinbane et al., 1997). Our observations of prolonged tachycardia in saline- and DZP-treated animals suggests that TIC may be implicated in our animals. There are multiple pathological changes observed in TIC and our study replicates several of these features (Khasnis et al., 2005). Haemodynamic changes are reported to occur as early as 24 hours following pacing in humans and in animal models (Shinbane et al., 1997). Haemodynamic parameters have been shown to normalise within 48 hours of pacing termination, however ECG changes (e.g. QT prolongation) and structural changes (e.g. fibrosis) may persist for weeks to months (Ellis & Josephson, 2013). Extending our study length to several weeks would allow investigation into whether these changes are reversible. As TIC is a reversible condition, early intervention is important (Khasnis et al., 2005). TIC is treated according to the underlying arrhythmia, and β-blockers or calcium channel blockers may be indicated for rate control (Lishmanov et al., 2010; Nakazato, 2002).

4.4.2 Stress cardiomyopathy

SCM is a cardiac disorder characterised by acute left ventricular dysfunction, often triggered following exposure to an emotional or physical stressor (Wittstein, 2012). SCM has been reported to occur following seizures, with this prevalence estimated at 1% (Dupuis et al., 2012; Legriel et al., 2008; Lemke et al., 2008; Sakuragi et al., 2007; Stöllberger et al., 2009; Stöllberger et al., 2011). Lyon et al. (2008) proposed that the stressful situation causes a robust release of catecholamines (particularly adrenaline), which may induce epicardial vasospasm, hyperinotropic responses, β₂-G, to β₂-G, switch in the apex, and direct cardiotoxic effects on cardiomyocytes. Hearts of SCM patients exhibits features of catecholamine cardiotoxicity, such as inflammatory cell infiltration, cardiomyocyte apoptosis and necrosis (reviewed by Wittstein, 2012; Wittstein et al., 2005). Furthermore, SCM often presents with QT prolongation, mild cardiac troponin elevation, raised plasma catecholamine levels, sympathetic dominance, and increased stress hormone levels (reviewed by Nef et al., 2010; Wittstein et al., 2005), all of which were demonstrated, in this thesis, or in separate studies of these animals (Read, 2014). Therefore, it appears that seizure induction by KA in our animals may precipitate a cardiomyopathy mirroring SCM.
Management of SCM can include use of β-blockers, although an animal model of adrenaline-induced SCM found that β₁ blockade with bisoprolol has little effect on apical (β₂-Gᵢ) function whilst exerting expected negative inotropic effects in the basal myocardium (β₁-Gₛ) (Paur et al., 2012). PDE inhibitors would be contraindicated in SCM patients, as the increased cAMP induced by adrenergic stimulation would exacerbate cardiac dysfunction (Paur et al., 2012). This suggests that if a SCM-type disorder is occurring in our rats, DZP treatment may be exacerbating the cardiac injury.

4.5 Conclusions

This study demonstrates that intrahippocampal KA-induced seizures are associated with significant tachycardia, QTc prolongation, and cardiac injury. β₁ blockade with atenolol, alone and in combination with DZP, was found to effectively reduce heart rate and QTc intervals following KA-induced seizures, and prevent all markers of cardiac damage. Disappointingly, DZP treatment alone did not attenuate the seizure-associated cardiac changes, and did not provide any cardioprotection. This may be due to DZP exerting central or direct cardiac effects including tachycardia, and suggests that its use in seizure control should be reconsidered in regards to potential cardiac effects. The results of this study imply that atenolol should strongly be considered as a cardioprotective adjunct therapy in seizure management.
Figure 4.3: Simplified theoretical mechanism of seizure-induced cardiomyopathy, and the effects of atenolol and diazepam intervention. The seizure causes activation of central sympathetic centres, leading to catecholamine release onto $\alpha_1$ adrenergic receptors causing increased blood pressure (BP), and onto $\beta_1$ adrenergic receptors causing increased heart rate (HR) and apoptosis. Tachycardia can cause myocardial ischaemia with ensuing cell death, cardiac troponin I ($cTnI$) release, and remodelling, leading to impaired cardiac function. Seizures may also induce respiratory dysfunction, leading to decreased oxygen saturation ($SpO_2$). The $\beta_1$ receptor antagonist, atenolol, is postulated to prevent excess sympathetic stimulation of cardiac $\beta_1$ receptors, and therefore prevents $\beta_1$-mediated apoptosis, tachycardia, and downstream cardiac remodelling. Diazepam is effective in attenuating the seizure, but may induce tachycardia, by increasing central sympathetic outflow; alternatively, diazepam is reported to potentiate catecholamine effects on cardiomyocyte adrenergic receptors, or potentially independent of $\beta_1$ receptors by translocator protein (TSPO) receptor binding or directly effecting $Ca^{2+}$ handling in the cardiomyocyte. TSPO and changes in $Ca^{2+}$ handling are also implicated in cardiomyocyte cell death.

4.6 Clinical Implications

This study warrants further clinical studies investigating cardioprotective therapy in epileptics at high risk of adverse cardiac events. In particular, patients presenting to the emergency department with seizures or SE with accompanying tachycardia should be administered a rate-control drug as soon as possible to prevent any potential cardiac damage from excessive tachycardia and sympathetic stimulation. Current literature suggests that DZP potentiates the cardiac effects of cAMP producing agents (i.e. NA, adrenaline) (Carmen Collado et al., 1998; Juan-Fita et al., 2003). In a setting of seizures whereby NA and adrenaline levels are...
significantly increased, DZP hypothetically would further exaggerate the cardiostimulatory effects of catecholamines on the heart. DZP-treated animals did exhibit increased HR’s over saline-treated animals at 24 and 48 hours post-seizure, supporting this theory. We also did frequently observe non-significant increases in several parameters (HR, QTc interval, BP, SpO₂, cTnI, etc.) of DZP-treated animals compared to saline-treated animals, suggesting DZP may have been exacerbating the cardiac injury induced by seizures. Furthermore, DZP treatment was also possibly implicated in the deaths of the SUDEP rat (DZP-treated) and the rat that developed HF (combination-treated). Further investigation is required to elucidate whether these cardiac effects of DZP may be putting patients at risk of cardiovascular complications. In addition, all AED’s, including other benzodiazepines, should be thoroughly screened for potential cardiac adverse effects, as these may be implicated in potentiating cardiac injury during seizures. Atenolol was very effective in this study in preventing seizure-induced cardiac damage. As atenolol is already clinically indicated for rate control, and as it is generally well tolerated, atenolol should be considered for use in patients with epilepsy and SE for preventing excessive sympathetic stimulation on cardiac β₁ receptors (Frishman & Saunders, 2011). Whilst atenolol-treatment by itself was cardioprotective in this study, it does not imply that atenolol should be used as a monotherapy in epilepsy and seizure management. In this study, atenolol demonstrated no anticonvulsant effects. Additionally, as failure to rapidly control seizures is associated with an increased mortality rate, it implies an effective AED is required for SE management (Logroscino et al., 2002). In cases of drug-refractory epilepsy, atenolol may be of benefit in reducing cardiac damage due to uncontrollable seizures. However, the contraindication of β-blockers in bradycardia implies that atenolol treatment may not be suitable for patients whose seizures are associated with these cardiac changes (López-Sendó et al., 2004). This may also explain the sudden death of the rat following atenolol intervention, with the negative inotropic actions of atenolol possibly producing severe hypotension and reduced cardiac output, leading to death. This contraindication is a potential issue for prophylactic atenolol treatment in epileptics, as it is likely not possible to predict whether a seizure will elicit tachycardic or bradycardic changes.

4.7 Limitations

4.7.1 Intrahippocampal seizure model

There are several limitations to our intrahippocampal KA model. Whilst the insertion of the drug delivery cannula may be considered to breach the BBB, a recent study by our group demonstrated no increase in BBB permeation in control cannula animals compared to non-
cannulated animals, suggesting this was not an issue (unpublished data). ECG/EEG parameters could not be monitored using transmitter implantation in the 14 days post-KA animal group, as animal growth over the 3 week period from surgery to termination, would have affected electrode wire placement. Another limitation of this study is that these results can only be applied to the intrahippocampal KA seizure model, and not to other seizure models (e.g. pilocarpine model).

4.7.2 Experimental design

This study specifically examined the effects of intervention on seizure-induced cardiac changes, with the KA-saline group classed as the control-treatment group. Consequently we did not include control groups for the drug treatments in naïve animals. However, our group had carried out intrahippocampal saline-controls and atenolol-controls as part of a separate study (Read, 2014). This thesis project originally included \( n = 8 \) rats per 7-day treatment group, however 3 animals were excluded for not developing seizures, and a further 3 animals died prematurely, therefore numbers ranged from \( n = 5 \) – 8 per group, limiting statistical power.

4.7.3 Molecular studies

The TUNEL assay for apoptosis is reported to be limited in its specificity for labelling apoptotic cells, and may additionally label necrotic cells in some instances (Kung et al., 2011).

4.8 Future Directions

4.8.1 Animal studies

- Carry out continuous 48 hour behavioural video monitoring, to allow more concise observations of seizure behaviours.
- Extend the study period to 3 months, in order to allow development of spontaneous recurrent seizures, as a chronic model of epilepsy-related cardiac changes (Powell et al., 2014). Whilst these rats will not be able to have transmitters implanted, echocardiograph analysis would allow effective functional studies.
- Examine the cardiac effects of our DZP dosing regime in naïve animals, to investigate whether DZP induces cardiac injury independent of seizure activity.
- Pre-treat animals with prophylactic atenolol in combination with an AED, as a study on cardioprotection, relevant to clinical epilepsy management of recurrent seizures.
• Examine other AED’s; e.g. valproic acid or carbamazepine, clinically relevant in the management of epilepsy.

• Examine the third generation β-blocker, carvedilol (α₁, β₁ and β₂ antagonist), which also exerts peripheral vasodilatory effects to reduce BP alterations (Gentles, 2009).

4.8.2 Molecular studies

• Perform double-immunohistochemistry labelling for apoptotic cardiomyocytes, for example with anti-phalloidin (Pattison et al., 2008).

• Further investigate the cardiomyocyte cell death; e.g. apoptosis using caspase-3; necrosis via in vivo administration of anti-myosin antibody (Goldspink et al., 2004).

• Measure sympathetic cardiac activity in animals with intervention treatments, by measuring plasma NA levels or sympathetic nerve recordings (Esler & Kaye, 2000).

• Examine myocardial levels of proteins by western blot or immunohistochemical methods, for example, β₁ and β₂ adrenergic receptors, LTCC’s, Kv4.2 channels, HCN channels (Bealer et al., 2010; Ludwig et al., 2003).

• Further examine the inflammatory response by looking at other cell types (e.g. neutrophils) or cytokines involved (e.g. interleukins) (Frangogiannis, 2012).
References


