Therapeutic Approaches in the Attenuation of
Seizure-Induced Cardiomyopathy

Anastasia Alexeevna Andreianova

A thesis submitted for the degree of Master of Science at the
University of Otago, Dunedin, New Zealand

February 2011
ABSTRACT

Single subcutaneous administration of Kainic acid (KA) in the rat produces significant levels of seizure activity, including head tremors, salivation and tonic-clonic convulsions. Using electrophysiological quantitative techniques which measure electroencephalographic (EEG) as well as electrocardiographic (ECG) trace activity following KA administration, the effects of seizure activity on the function of the heart were assessed over a 48 hour period. In addition, histopathological analysis was carried out in order to determine whether the ongoing seizure activity produced significant changes in ventricular myocardium indicative of irreversible cardiomyopathy. In order to determine the potential mechanism of action of KA-induced cardiac damage, a further two animal groups were examined. The groups consisted of animals pretreated with either atenolol or clonidine. The two different drugs were used in order to isolate systems involved in cardiac damage, where atenolol acts specifically in the periphery, while clonidine is known to act in the central nervous system. Analysis of EEG and concomitant ECG traces, during and following seizure activity demonstrated significant changes in heart rate (HR) as well as associated HR parameters compared to baseline. Upon further histological observations it was apparent that at 48 hours following KA administration, ischaemia was present as well as evidence of inflammatory cell infiltration, tissue tearing and oedema compared to saline treated animals. Further assessment of pretreated animal groups lead to the conclusion that atenolol was not protective against KA-induced cardiac damage in the rat while clonidine was. These findings propose that the mechanism by which KA-induced seizure activity results in cardiomyopathy is through modulation of brain centres associated with cardiac control, as opposed to KA binding to peripheral cardiac receptors as previously suggested.
ACKNOWLEDGMENTS

I would like to say a huge thank you to my supervisors Dr Ivan Sammut and Dr Steve Kerr. To embark on such a huge journey of knowledge and understanding would not have been possible without your immense wisdom in your specialist fields as well as your constant support and encouragement. A massive thank you to Dr John Schofield and the Animal Welfare staffs who constantly worked with me to perfect surgery practises and enhance animal care. Thanks to all my colleagues in the neuropharmacology and cardiopharmacology labs who not only taught me the techniques that allowed me to complete my study successfully but who were also there in my times of need. Finally I would like to thank my family and friends, who stuck by me and kept me sane when the end appeared impossible, your constant support allowed me to remain positive even in the worst of times.
TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. ii
ACKNOWLEDGMENTS ................................................................................................................ iii
TABLE OF CONTENTS ................................................................................................................. iv
LIST OF FIGURES & TABLES ...................................................................................................... vi
LIST OF ABBREVIATIONS ........................................................................................................ viii
INTRODUCTION .......................................................................................................................... 0
  Introduction to Seizures .................................................................................................................. 1
  Seizure Types .............................................................................................................................. 1
  Cellular Basis of Seizure Activity ............................................................................................... 3
  Kainic Acid and Seizure Induction .............................................................................................. 5
  Epilepsy and S.U.D.E.P. .............................................................................................................. 7
  ANS Involvement During Seizure Activity (LSP) ...................................................................... 9
  Myocardial Damage in Response to Seizure .......................................................................... 13
  Insular Cortex Involvement in ANS Modulation .................................................................... 16
  SUDEP and the Use of AEDs .................................................................................................... 17
  Preventative Measures in SUDEP .......................................................................................... 18
  Aims and Hypothesis ................................................................................................................ 20
METHODS ...................................................................................................................................... 22
  Materials ................................................................................................................................... 23
  Animal Care .............................................................................................................................. 23
  Experimental Protocol ............................................................................................................. 23
  Animal Surgery (Electrode Placement) ................................................................................... 24
  Animal Dosing .......................................................................................................................... 27
    Atenolol .................................................................................................................................. 27
    Clonidine ............................................................................................................................... 27
    Kainic Acid ............................................................................................................................ 28
  Behavioral Analysis .................................................................................................................. 28
  EEG and ECG Recordings ........................................................................................................ 29
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac Isolation and Perfusion-Fixation</td>
<td>30</td>
</tr>
<tr>
<td>Histology</td>
<td>31</td>
</tr>
<tr>
<td>Statistical Analysis</td>
<td>31</td>
</tr>
<tr>
<td><strong>RESULTS</strong></td>
<td>32</td>
</tr>
<tr>
<td>Morbidity and Mortality</td>
<td>33</td>
</tr>
<tr>
<td>Seizure Activity Following KA Administration</td>
<td>33</td>
</tr>
<tr>
<td>Seizure Related Behavioral Observations</td>
<td>34</td>
</tr>
<tr>
<td>ECG-derived Heart Rate Responses: Bradycardia</td>
<td>36</td>
</tr>
<tr>
<td>ECG-Derived Heart Rate Responses: Tachycardia</td>
<td>37</td>
</tr>
<tr>
<td>Individual Animal ECG Observations</td>
<td>40</td>
</tr>
<tr>
<td>Heart Rate Parameter Observations</td>
<td>43</td>
</tr>
<tr>
<td>Individual ECG Observations (Ictal Period)</td>
<td>47</td>
</tr>
<tr>
<td>Histology</td>
<td>54</td>
</tr>
<tr>
<td><strong>DISCUSSION</strong></td>
<td>55</td>
</tr>
<tr>
<td>KA and Seizures</td>
<td>56</td>
</tr>
<tr>
<td>KA induced Bradycardia</td>
<td>57</td>
</tr>
<tr>
<td>Tachycardia Following KA</td>
<td>64</td>
</tr>
<tr>
<td>HR Parameter Changes</td>
<td>65</td>
</tr>
<tr>
<td>Histological Observations</td>
<td>70</td>
</tr>
<tr>
<td>Limitations to the Experiment</td>
<td>75</td>
</tr>
<tr>
<td>Conclusion</td>
<td>76</td>
</tr>
<tr>
<td><strong>REFERENCES</strong></td>
<td>77</td>
</tr>
</tbody>
</table>
LIST OF FIGURES & TABLES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chemical and structural similarities between domoic, kainic and glutamic acids</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Electrical conduction system of the heart</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>Surgical procedure of transmitter implantation</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>EEG recordings during seizure activity</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>Averaged sum of behavioral seizure scores</td>
<td>35</td>
</tr>
<tr>
<td>6</td>
<td>Time to onset of initial level 4 seizure behaviour</td>
<td>35</td>
</tr>
<tr>
<td>7</td>
<td>EEG and ECG recordings at 15ms intervals in animal treated with KA</td>
<td>36</td>
</tr>
<tr>
<td>8</td>
<td>Averaged HR comparing treated groups</td>
<td>38</td>
</tr>
<tr>
<td>9</td>
<td>Individual ECG trace of an animal’s HR</td>
<td>39</td>
</tr>
<tr>
<td>10</td>
<td>P wave disruption</td>
<td>41</td>
</tr>
<tr>
<td>11</td>
<td>HR variation vs. seizure activity</td>
<td>42</td>
</tr>
<tr>
<td>12</td>
<td>RR interval values across specific time points comparing saline to treated groups</td>
<td>43</td>
</tr>
<tr>
<td>13</td>
<td>QTc lengths compared across treatment groups</td>
<td>45</td>
</tr>
<tr>
<td>14</td>
<td>HR abnormalities</td>
<td>48</td>
</tr>
<tr>
<td>15</td>
<td>Individual data for saline-control rat, HR, spike frequency and behaviour</td>
<td>49</td>
</tr>
<tr>
<td>16</td>
<td>KA-untreated rat, EEG trace, behavioral scores and spike frequency</td>
<td>50</td>
</tr>
<tr>
<td>17</td>
<td>Atenolol-treated rat, EEG trace, spike frequency and behavioral scores</td>
<td>51</td>
</tr>
<tr>
<td>18</td>
<td>Clonidine-treated rat, visual representation of HR, spike frequency and behaviour</td>
<td>52</td>
</tr>
<tr>
<td>19</td>
<td>Histopathological changes in apex and level 2 of heart tissue</td>
<td>53</td>
</tr>
<tr>
<td>20</td>
<td>Potential action of systemic KA injection</td>
<td>63</td>
</tr>
<tr>
<td>21</td>
<td>Example of bradycardia and skipped beats</td>
<td>69</td>
</tr>
<tr>
<td>22</td>
<td>Two mechanisms describing potential cardiac effects in response to KA administration</td>
<td>74</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Summary of cardiovascular risk factors</td>
<td>15</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiepileptic drugs</td>
<td>AEDs</td>
</tr>
<tr>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate</td>
<td>AMPA</td>
</tr>
<tr>
<td>Autonomic Nervous System</td>
<td>ANS</td>
</tr>
<tr>
<td>Atrioventricular Node</td>
<td>AV</td>
</tr>
<tr>
<td>Beats per minute</td>
<td>bmp</td>
</tr>
<tr>
<td>Cyclic AMP</td>
<td>cAMP</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>CBZ</td>
</tr>
<tr>
<td>Circling</td>
<td>C</td>
</tr>
<tr>
<td>Central Nervous System</td>
<td>CNS</td>
</tr>
<tr>
<td>Corrected QT</td>
<td>cQT</td>
</tr>
<tr>
<td>Tonic-Clonic Convulsions</td>
<td>CTC</td>
</tr>
<tr>
<td>Cortical Epileptiform Activity</td>
<td>EA</td>
</tr>
<tr>
<td>Electrocardiogram</td>
<td>ECG</td>
</tr>
<tr>
<td>Electroencephalogram</td>
<td>EEG</td>
</tr>
<tr>
<td>Hind Limb Extension</td>
<td>EXT</td>
</tr>
<tr>
<td>Freezing</td>
<td>FRZ</td>
</tr>
<tr>
<td>γ-aminobutyric acid</td>
<td>GABA</td>
</tr>
<tr>
<td>(1)-(4-aminophenyl)-3-acetyl-4-methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine</td>
<td>GYKI-53773</td>
</tr>
<tr>
<td>Haemotoxylin and Eosin</td>
<td>H &amp; E</td>
</tr>
<tr>
<td>Heart Rate</td>
<td>HR</td>
</tr>
<tr>
<td>Head Tremors</td>
<td>HT</td>
</tr>
<tr>
<td>Hyperactivity</td>
<td>HYP</td>
</tr>
<tr>
<td>‘funny current’</td>
<td>If</td>
</tr>
<tr>
<td>Term</td>
<td>Abbreviation</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Intra-hippocampal</td>
<td>ih</td>
</tr>
<tr>
<td>Intraperitoneal</td>
<td>ip</td>
</tr>
<tr>
<td>Kainic acid</td>
<td>KA</td>
</tr>
<tr>
<td>K⁺ rectifier channel</td>
<td>Kᵅᵣ</td>
</tr>
<tr>
<td>Locus Coeruleus</td>
<td>LC</td>
</tr>
<tr>
<td>Lock-step phenomenon</td>
<td>LSP</td>
</tr>
<tr>
<td>Mastication</td>
<td>M1</td>
</tr>
<tr>
<td>Myoclonic Jerks</td>
<td>MJ</td>
</tr>
<tr>
<td>Noradrenalin</td>
<td>NA</td>
</tr>
<tr>
<td>2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[f]quinoxaline</td>
<td>NBQX</td>
</tr>
<tr>
<td>N-methyl-D-aspartate</td>
<td>NMDA</td>
</tr>
<tr>
<td>Nucleus of the Solitary Tract</td>
<td>NTS</td>
</tr>
<tr>
<td>Parabrachial Nucleus</td>
<td>PB</td>
</tr>
<tr>
<td>Postganglionic Cardiac Sympathetic Discharge</td>
<td>PCSD</td>
</tr>
<tr>
<td>Renin-Angiotensin System</td>
<td>RAS</td>
</tr>
<tr>
<td>Rearing</td>
<td>REAR</td>
</tr>
<tr>
<td>Reticular Formation</td>
<td>RF</td>
</tr>
<tr>
<td>Sinoatrial Node</td>
<td>SA</td>
</tr>
<tr>
<td>Scratching</td>
<td>SC</td>
</tr>
<tr>
<td>Salivation</td>
<td>SAL</td>
</tr>
<tr>
<td>Standard Error of the Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Sudden, unexpected, nontraumatic and nondrowning death in an individual with epilepsy</td>
<td>SUDEP</td>
</tr>
<tr>
<td>Wet-Dog Shakes</td>
<td>WDS</td>
</tr>
<tr>
<td>Vagus Nerve Stimulation</td>
<td>VNS</td>
</tr>
</tbody>
</table>
INTRODUCTION
Introduction to Seizures

The word ‘seizure’ refers to a momentary change of behavior occurring as a result of synchronous, disordered or rhythmic firing of a combined population of excitatory central nervous system (CNS) neurons (McNamara, 1994). In particular, ‘epilepsy’ is defined as a disorder of the brain which results in periodic and unpredictable occurrence of seizures that varies both in duration and severity. Following the Rochester Epidemiology Study, the causes of epilepsy, onset of, and ongoing seizure activity have been associated with a number of factors (Annegers et al., 1996). In their unique population-based evaluation, it was shown that the main predisposing factors contributing to epilepsy included traumatic brain injuries, brain tumors, central nervous system infections, cerebrovascular disease, neurodegenerative disorders as well as perinatal insults. The controversial factors of inheritance and environmental factors such as toxins were also considered and were reasoned to have a considerable influence in causing seizures and epilepsy.

Seizure Types

Seizures can exist in many different forms. They can be convulsive or non-convulsive during which the individual may experience complete loss of consciousness in some cases. The seizures may be tonic-clonic, generalized or partial, lasting for varying periods of time. Their effects may become evident in the involuntary muscle movement of the affected individual or may remain clinically asymptomatic without any observable motor effects while simultaneously showing considerable changes during EEG recordings (Delanty et al., 1998). Partial seizures arise in one specific part of the brain, most commonly the cortex, and they remain localized to that specific region during the entire
seizure period. These partial seizures can either be simple or complex, depending on the level of severity. Simple partial seizures can often go unnoticed or may present in an individual as a momentary daydream. The majority of complex partial seizures originate in the temporal lobe and can result in some degree of impairment of consciousness (McNamara, 1994), however these generally occur without any evident convulsive behavior. Generalized seizures on the other hand arise from one brain region and then continue to spread throughout the rest of the brain. This type of seizure activity can result in either myoclonic jerks, ensuing in a single surge of muscle activity or tonic-clonic convulsions involving sustained or prolonged muscle contraction. The behavioral manifestations that accompany seizures can be related back to the type of seizure and thus its origin. An excellent animal model used extensively to demonstrate the differing stages of seizure is the application of direct electrical stimulation to specific brain regions, evoking a particular response specific to the stimulated part of the brain, a method otherwise known as the kindling model of epilepsy (McNamara et al, 1985). The phenomenon of kindling is defined by McNamara, (1984), where repeated administration of non-convulsive electrical stimulation results in a gradual increase of seizure activity which eventuates into generalized seizures. The kindling model is considered representative of generalized seizures affecting both cortical hemispheres in patients. This transheisheric effect results in tonic-clonic convulsions and a complete loss of bodily function as muscle control is completely influenced by the motor cortex. Not only has this model allowed us to understand the direct connections associated with movement control but has also demonstrated how long term stimulation similar to that experienced by patients suffering chronic epilepsy, can influence synaptic plasticity in the
hippocampus (Represa et al., 1989; Morimoto et al., 2004), cerebral cortex and subcortex (Frost et al., 1988) as well as the thalamus (Bertram et al., 2001).

**Cellular Basis of Seizure Activity**

Fundamental methods used to define and analyse seizure activity involve electrical brain wave monitoring. This is achieved by recording rhythmic currents which are generated as a result of the intrinsic oscillatory properties of thalamic nuclei. The thalamic nuclei generate the rhythmic activity by receiving sensory inputs along with unspecific inputs from the cholinergic brain-stem-thalamic projections and monoaminergic projections which arise from the locus coeruleus (LC) (Lopes da Silva, 1991). As these neurons communicate, they produce feed-back and feed-forward signals by means of cell depolarisation enabling excitation and inhibition, which in turn results in the rhythmic activity observed using electroencephalogram (EEG) recordings. In the context of a diseased brain in which cellular integrity is often compromised, the resultant oscillations are disrupted in such a way as to produce certain patterns of activity that are indicative of a disruption of brain function, such as that of an ongoing seizure.

As mentioned previously, a seizure can result in response to a large number of influences, including direct damage to the neuronal cells as well as the effect of specific toxins on the body. The current general understanding is that this process can be explained as a lethal cascade of events occurring in the oscillating cells that produce the normal intrinsic rhythm. This cascade results in the opening of specific ion channels (Mody & Bradley, 1998), such as $K^+$ and also $Cl^-$ however, the most commonly recognised ion associated with damage is $Ca^{2+}$. The influx of $Ca^{2+}$ through the ligand-gated cation channels and
activation of secondary messengers results in neuronal cell death (Choi, 1987). As the cell is no longer able to perform normal function, synchronised cell firing can no longer take place, therefore resulting in abnormal brain function. For decades it has been recognised that glutamate, a naturally occurring excitatory amino acid is responsible for the deadly cellular cascade (Chapman, 2000). In the mammalian nervous system, glutamate has been established as the main excitatory neurotransmitter, and exerts its action through different types of glutamate receptors. As glutamate is released on to specific glutamate receptors, whether it is in response to pharmacological influence, poisoning or neuronal damage, the result is an over excitation of the neuron. The subsequent activation of secondary messengers which leads to excitotoxicity is thereby responsible for pathogenesis of the neuronal cell which leads to neurological disease (Choi et al., 1987; Delanty et al., 1998). There are two main types of glutamate receptors, ionotropic and metabotropic. Ionotropic receptors containing an integral ion channel, are further divided into three distinct groups; α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) glutamate receptors, while metabotropic receptors are coupled to G-proteins which stimulate the production of downstream cellular second messengers such as cyclic AMP (cAMP), (Ozawa et al., 1998). In their studies, Rackhade et al., (2008), used a rodent model of epilepsy to determine the role of AMPA receptors in seizure activity. This model focused on the knowledge that the neonatal brain is highly susceptible to seizure and will further predispose to long term CNS damage and epilepsy. They established that administration of AMPA receptor antagonists, NBQX (2,3-dihydroxy-6-nitro-7-sulfonylbenzo[f]quinoxaline), topiramate, or GYKI-53773 ((1)-1-(4-aminophenyl)-3-acetyl-4-
methyl-7,8-methylenedioxy-3,4-dihydro-5H-2,3-benzodiazepine) not only prevented long term seizure susceptibility and seizure-induced injury demonstrated in their previous experiments (Koh et al., 2004), but also reduced the potentiation, phosphorylation and kinase activation of the AMPA receptors, observed following seizure activity.

Another endogenous ligand that requires significant consideration is γ-aminobutyric acid or GABA. This is an inhibitory amino acid, which forms a vital part of neuronal communication responsible for the brain’s rhythmic activity. In a healthy brain the levels of both excitatory and inhibitory amino acids would find balance in optimising control. However, just as there can be an over excitation, the lack of inhibition ultimately results in the excitotoxicity of neuronal cells, thereby resulting in seizure induced cellular damage.

**Kainic Acid and Seizure Induction**

In 1987 there was an outbreak of shellfish poisoning in Canada. The reported case study by Teitelbaum et al., (1990); Perl et al., (1990), demonstrated that this was the result of ingesting contaminated mussels that contained an excitatory amino acid called domoic acid. The patients suffered from an array of symptoms including gastrointestinal upsets, a variety of arousal abnormalities which ranged from headaches to complete loss of consciousness and coma. However, more interestingly, they suffered from neurological disturbances following which, there was extensive memory loss and in three elderly patients the ingestion was lethal. Following analysis obtained from the autopsy report of all three of the deceased patients, Perl and co-workers observed neuronal necrosis and astrocytosis, which was found to be most prominent in the hippocampal and amygdala
cell layers. One of the patients went into a comatose state and in addition they experienced unilateral seizure activity without any evident EEG changes.

Domoic acid, kainic acid (KA) are structurally related to endogenously occurring glutamate and are capable of acting as ligands at glutamatergic receptors (Coyle, 1983), (Fig. 1). Both of these exogenous acid compounds, produce potent neurotoxic and excitotoxic effects on the neuronal cell (Herndon & Coyle, 1977). These effects were shown to occur as a result of KA acting as an agonist at the glutamate receptor. Furthermore, this receptor binding generated a non-desensitising or a slow-desensitising response to both AMPA and kainate receptors, compared to the rapid desensitising response produced by glutamate. This response is explained by the high affinity binding displayed by KA at the glutamatergic receptors and suggests an explanation for KA-produced neurotoxicity (Hampson & Manalo, 1998). Recent studies have explored the effects of KA administration at kainate receptors (for review see Vincent & Mulle, 2009). These studies further demonstrated the high affinity binding of KA at these receptor sites, which has previously been shown to be 40-fold more potent than the naturally occurring analogue L-glutamic acid (London & Coyle 1979), and the direct association with temporal lobe epilepsy and neuronal tissue damage. As well as the possible potentiation exerted at the glutamate receptors, other studies have also demonstrated that KA may act to decrease GABAergic inhibition, thus resulting in over excitation predisposing the animal to seizures (Dakshinamurti et al., 1991). The cascade of events leading to neuronal damage and potential seizure activity may therefore be explained by the action of KA at the excitatory neurotransmitter receptors. We have consequently employed an
established systemic-delivery protocol of KA-induced neurotoxicity to produce our model of epilepsy and seizure behaviour in rats (Tasker et al., 2010).

**Figure 1:** Schematic demonstrating the chemical and structural similarities between domoic, kainic and glutamic acids that allow the toxic algae to bind glutamate receptors.

**Epilepsy and S.U.D.E.P.**

As described previously, epilepsy is a debilitating illness that may cause the patient to experience different types of seizure activity throughout their lifetime. Epilepsy affects approximately 50 million people worldwide (Dua et al., 2006) increasing mortality rates in affected individuals by 2-3 times that of the general population, with higher risk ratios reported for those individuals suffering from chronic epilepsy. Of the epilepsy sufferers that are in the higher risk patient group, over 40% of deaths occur as a result of sudden, unexpected, nontraumatic and nondrowning death in an individual with epilepsy (SUDEP) (Tellez-Zenteno et al., 2005). The precise pathological mechanism associated with SUDEP has been extremely difficult to determine, as there has been as yet no definitive anatomical or toxicological explanation of the causes of death provided by post-mortem examination. It is thereby defined as a diagnosis of exclusion where no other diagnosis can be assigned as being the cause of death in that particular individual. The determining factors are applied when there is no obvious cause such as infection, trauma, pulmonary embolism or
myocardial infarct that has occurred in an otherwise healthy individual (Leestma et al., 1997). The lack of witnessed SUDEP episodes poses the question as to the precise number of SUDEP associated mortality rates. This uncertainty has put pressure on seizure-related research to determine the predisposing risk factors so as to form a basis for the development of effective therapeutic/preventative measures. So far, it has been established that the biggest risk factors that may be responsible for predisposing epileptic individuals to premature death are associated with seizure frequency. In a clinical case-control study, researchers (Nilsson et al., 1999) demonstrated that the relative risk of SUDEP increased with the amount of seizures patients had per year. The risk increased by 10% in patients who experienced 50 seizures in one year, compared to those who suffered two in the same time frame. The other risk factors discovered in the study were associated with an increase in the use of antiepileptic drugs, which will be discussed later, frequent changes in antiepileptic medication and sex differences, where the risk appears to increase in males. Although there is a lack of evidence supporting significant contributing factors that result in SUDEP, there are certain features that are observed in the individual during autopsy. The most commonly reported pathologies include pulmonary oedema, although not sufficient to cause mortality on its own, as well as increased lung, liver and cardiac weights (Earnest et al., 1992). Based on the autopsy findings as well as numerous human and animal studies that will be discussed later, it has been widely believed that there must be a relationship between seizure occurrence and a subsequent autonomic nervous system (ANS) activation, which results in the pathology associated with SUDEP. This relationship is otherwise known as the lock-step phenomenon (LSP) and is the occurrence of postganglionic cardiac sympathetic discharge.
(PCSD) and cortical epileptiform activity (EA) in a time-locked fashion (Stauffer et al., 1989).

**ANS Involvement During Seizure Activity (LSP)**

The ANS can be otherwise considered as the control centre of the body. It consists of a framework of both central and peripheral components; glial and neuronal cells, which allow the brain to communicate with the rest of the body as well as the body with the brain, relaying the information back and forth (Gabella, 2001). The central components of the ANS that originate in the brain consist of large nuclei and are located in the hypothalamus, these neurons are connected to long aggregates of nuclei in the brain stem and spinal cord. As the ANS neurons leave the central nervous system they send nerve projections into the periphery, where finally the postganglionic nerve terminals synapse onto visceral tissue containing differing receptor types. There are two main components that constitute the ANS, these are the sympathetic and the parasympathetic pathways which depending on the type of tissue, synapse at specific receptor types activated by that specific system. This activation results in a working relationship of both pathways to oppose and enhance each other in a co-operative manner in controlling the vital organs of the body such as the heart, lungs, liver and kidneys. When the sympathetic system is activated, its preganglionic neurons release vesicles containing acetylcholine onto nicotinic receptor sites. This activation results in the release of noradrenalin onto adrenergic receptors located on skeletal, smooth and cardiac muscle. Specifically in the heart, activation of the $\beta_1$-adrenergic receptors results in an increased ionotropic (ventricular contractile force) as well as increased chronotropic (beating rate) cardiac response. The sympathetic system may also increase the activity of the heart by acting on the adrenal medulla, thus causing a release of adrenalin into the blood stream and a
subsequent activation of adrenergic receptors in the cardiac muscle. The parasympathetic system works in an opposing manner. The postganglionic neurons release acetylcholine onto muscarinic receptor terminals, which results in a negative ionotropic and chronotropic response. Without the involvement of the parasympathetic system, the human heart would beat at an average resting heart rate of 100 beats per minute (bpm), thereby the parasympathetic system acts under physiological conditions to reduce the workload on the heart, thus protecting it from long term damage.

Based on our current understanding of the role of the ANS, it is logical to assume that seizure activity spreading throughout many brain regions could have a major impact on the ANS control centres, thus resulting in changes in the downstream projections which influence normal organ function such as cardiovascular function. There is a large amount of evidence supporting the involvement of the ANS during seizure activity with attention paid to the involvement of cardiovascular abnormalities that occur in both animal models as well as humans, many of the documented cardiovascular effects are summarised in table 1.

In a case-controlled study where 20 patients were implanted with ECG loop-recorders, researchers (Rugg-Gunn et al., 2004), found that in 16 patients, heart rate exceeded 100 bpm during habitual seizure activity. A subsequent study (O'Regan & Brown, 2005), demonstrated significant fluctuations in cardiac parameters such as an increase or a decrease in heart rate that were present in 50% of patients who had generalized seizures. In most human studies it has been very evident that the most common occurrence observed during seizure activity is an increase in heart rate or tachycardia (Nei, 2009). These cardiac changes may also lead to development of asystole and arrhythmias, which may become fatal without intervention and are thus suspected to be centrally implicated in SUDEP. Other studies
(Espinosa et al., 2009) focusing on the electrical system of the heart, have indicated how a disruption of HR occurring specifically within the cardiac rhythm, particularly concerning the conduction pathway, can also have lethal consequences. The electrocardiogram (ECG) is an accurate depiction that demonstrates the electrical pathway of one complete heartbeat. Each spike demonstrates the opening and closing of specific ion channels which are a result of depolarising current travelling through the heart, beginning at the sinoatrial node (SA), spreading through the heart, to the atrioventricular node (AV) and ending at the ventricular myocardium, following which depolarisation begins again (refer to figure 2).

![Image 1](image1.png)

**Figure 2:** Schematic diagram representing the electrical system of the heart and its presentation on an electrocardiogram.

When considering the pathology of the heart and the possibility of cardiac damage, it is important to concentrate on the specific regions associated with the conduction pathway, and the concomitant trace of the ECG wave. The P wave represents depolarisation of the atrium
and the concomitant PR interval is indicative of propagation of the electrical current to the AV node. The QRS interval demonstrates depolarisation of the ventricles. Although cardiac abnormalities can be depicted from any region within the ECG, many studies (Chugh et al., 2009) focus on the QT interval, which can be seen to be either prolonged or shortened, depending on the pathology, and indicating that the patient could be at risk of suffering a fatal arrhythmia (Brugada et al., 2004). QT interval is used as a clinical index when assessing primary cardiac arrest in heart disease (Whitsel et al., 2001), QT dispersion in particular is used in order to predict future cardiac events following a myocardial infarction (Zabel et al., 1998), and it is also one of the main parameters used to determine early cardiac morbidity and mortality following ischaemic stroke (Prosser et al., 2007). QT elongation has been associated with a very serious cardiac condition known as long QT syndrome, where it has been known to predispose individuals to ventricular tachycardia, torsade de pointes and sudden death (Khan, 2002). QT interval alterations have been associated with seizure events. This has been evident among children as well as adults, for example, in a study of seizure episodes in twenty children and adolescents, (Mayer et al., 2004; Surges et al., 2010), researchers found that along with ictal tachycardia observed in 98% of temporal lobe seizures, QT interval shortening occurred in 17 of those patients, while QT prolongation occurred in 3 patients. QT elongation is of particular concern when determining any potential risk to cardiac function as it is an established prognostic marker in the development of cardiac arrhythmias leading to ventricular fibrillation and sudden death. Recently however, it is becoming evident that a shortened QT may also result in such a disruption of the ventricular repolarisation as to cause an arrhythmic event that can arrest the heart (Gaita et al., 2003).
Given that the QT interval is a large representative measure of the cardiac cycle, it is difficult to accurately measure. This issue arises from the large variability relating to different components that comprise this measure, particularly due to the imprecision in identifying the end of the T wave, and its correlation to changes in heart rate (HR) (Moss, 1993). As a result of this, a corrected form of QT measurement (QTc) has been developed using a number of different regression formulas that take into account the relationship between QT and RR interval. Based on past research requiring the use of QTc, we decided to use the Bazzett formula (Sarma et al., 1984), to determine QT in the current study. This correction is expressed as follows:

\[ \text{QTc} = \frac{\text{QT}}{\text{RR}^{1/2}} \]

**Myocardial Damage in Response to Seizure**

As in any other living cell of a multicellular organism, the myocardial cells require oxygen in order to maintain normal function and ensure survival. The mitochondria are central at maintaining this oxygen level in the cells of the heart. The cardiomyocyte differs as it is one of the only cells that cannot maintain energy stores via anaerobic glycolysis if oxygen stores are depleted (Piper et al., 1994), thereby making it especially susceptible to damage during conditions of low oxygen such as those that are exhibited in the heart during periods of high workload. Therefore in order to understand the potential damage associated with disrupted cardiac function, as that proposed to occur following seizure, it is essential to focus on the oxidative energy production of the mitochondria. The mitochondrion is a complex energy storage device that under normal respiratory conditions acts to synthesise ATP from ADP by producing a membrane potential and a proton gradient through the transport of electrons in
its inner membrane. The ATP is a vital component in driving ion channels such as the \( \text{Na}^{2+}/\text{K}^+ \) ATPase which is essential in maintaining the resting membrane potential (Cortassa, *et al*., 2003). It is also important when considering the spontaneous contraction of the myocardial cell at the electrical centres like the SA and AV nodes, and the Purkinje fibres. At these electrical locations a collaboration of ion channels make up the very important ‘funny current’ (\( \text{I}_\text{f} \)) that allows the cell to depolarise through spontaneous contraction arising from leaky current (DiFrancesco, 1993). Therefore in situations when the heart is working beyond its capacity, especially during long periods of tachycardia and ventricular tachycardia, the oxygen production of the mitochondria will be insufficient to supply the demand of all the cells. The general concept concerning damage resulting from reduced oxygen levels is further enhanced through the activation of apoptotic factors that encapsulate the nucleus and promote cell death (Suematsu *et al*., 2002).

There is also a large amount of evidence supporting the involvement of the renin-angiotensin system (RAS) in the development of cardiac hypertrophy in hypertensive crisis (Patel & Mitsnefes, 2005). The local synthesis of the RAS components in the heart and their constant interplay with circulating RAS, mechanical stretch and the involvement of the sympathetic nervous system are all pivotal factors involved in fibrosis, remodelling and cardiac hypertrophy (Bader, 2002). Although this is normally a long-term process that eventually results in the significant progressive changes, it is possible to speculate that these factors indeed would be occurring in an individual that suffers from epilepsy over their entire lifespan. The associated ANS modulation that is proposed to occur (LSP), along with potential variation in HR, may be presenting similar conditions that ultimately result in progressive cardiac damage, like that seen in cardiac failure and hypertension.
<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Seizure Types</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ictal Tachycardia</td>
<td>- TLE (complex partial seizures)</td>
<td>- In 26 patients 92% experienced elevated HR (Blumhardt et al., 1986), 72 recorded seizures, 98% ictal tachycardia (Mayer et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>- Lateralized and generalized seizures</td>
<td>- 45 observed patients, ictal tachycardia observed in 96% (Keilson et al., 1989), (Opherk et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>- SUDEP occurred during seizure</td>
<td>- 14/16 patients died in their sleep, result of SUDEP, experienced HR elevation to 146 bpm. (Nei et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>- Unilateral TLE</td>
<td>- Occurred in 27 seizures of 5 patients, HR increase correlated with volume of brain involved. (Epstein et al., 1992).</td>
</tr>
<tr>
<td>Bradycardia</td>
<td>- Complex partial seizures and generalized tonic clonic</td>
<td>- 7 patients experienced transient bradycardia of 6-42 bpm, (Nashef et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>- Left temporal and frontal lobe seizures</td>
<td>- 3 cases of ictal bradycardia, specific to left hemisphere. (Tinuper et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>- Epilepsy with syncope, TLE</td>
<td>- 23 patients observed, ranging in years of age with a 5:1 ratio of males to females, accompanied by asystole (Reeves et al., 1996).</td>
</tr>
<tr>
<td>QT - elongation/</td>
<td>- Generalized tonic clonic seizures</td>
<td>- QTc shortening presented in 17 patients, QTc lengthening in 3 patients (Surges et al.).</td>
</tr>
<tr>
<td>-shortening</td>
<td>- Epileptiform, generalized tonic-clonic</td>
<td>- Adjusted QTc lengthening more pronounced in patients who have later died from SUDEP. (Tavernor et al., 1996).</td>
</tr>
<tr>
<td>Fatal arrhythmia</td>
<td>- Seizure resulting in SUDEP</td>
<td>- Patient with a witnessed seizure who was undergoing ECG monitoring. Suffered fatal arrhythmia during seizure, no anatomical cause of death. (Dasheiff &amp; Dickinson, 1986)</td>
</tr>
</tbody>
</table>

Table 1: Summary of the cardiovascular risk factors associated with certain seizure types and the concomitant observations obtained.
Insular Cortex Involvement in ANS Modulation

It has recently been recognised that cardiovascular disturbances are not only attributed to the medulla control centres where ANS afferents originate, but also to higher regions of the brain such as the insular cortex. Although clinical evidence (Lathers et al., 2008) supports the involvement of the cerebral cortex in the development of cardiovascular disturbances during and following seizure activity, there is little evidence that identifies this connection directly. The insular cortex however, has been proposed to serve as the chronotropic centre involved in the direct modulation of heart rate. In their study, (Oppenheimer & Cechetto, 1990), reproducibly demonstrated the cardiac chronotropic sites in 37 anaesthetised rats, where tachycardia was produced as a result of stimulation of the rostral posterior insula while the bradycardic response was evoked from the caudal posterior insula. The chronotropic effects were evoked directly and with no other ANS consequences such as correlated changes in blood pressure or respiratory rate. Thereby, the cardiovascular disturbances that have been proposed to occur and be responsible in SUDEP may be attributed to electrical or seizure activity originating from the insular cortex based on its extensive autonomic and limbic connectivity. Further investigation into the effects of insular cortex stimulation and the subsequent response of the heart has also been elicited through injection of an excitatory amino acid. Unilateral administration of KA into the insular pressor area was found to result in topographically specific elevations in arterial pressure and tachycardia (Ruggiero et al., 1987). Inversely, there is further evidence that links the importance of the insular cortex and autonomic function, as Meyer and co-workers (Meyer et al., 2004), were able to demonstrate from the clinical analysis of patients, who had experienced ischaemic insular infarction compared to non-insular infarction. Based on their assessment of adrenaline and noradrenalin
blood concentration levels as well as the quantification of blood pressure and heart rate effects they found that sympathetic activity was significantly higher in patients who suffered infarction in the insular region. This reduction in autonomic regulation resulting from ischaemic damage, along with evoked cardiovascular responses following microstimulation of the same brain region further implicates the insular cortex and the potential for autonomic dysfunction in an individual with epilepsy that could be the underlying cause of sudden death.

**SUDEP and the Use of AEDs**

Based on the current knowledge of the associated risk factors and prevalence of SUDEP, this study will assess possible pharmacological measures that may be taken, in order to establish a novel therapeutic measure to prevent the occurrence of sudden unexpected death in epilepsy.

In respect to the use of antiepileptic drugs (AEDs), and the proposed effects on the ANS seen during seizures, it is necessary to consider the specific effects that AEDs have on the cardiovascular system. In a study performed by Persson and colleagues, the effects of a commonly used AED, carbamazepine (CBZ) was assessed on cardiovascular function, in newly diagnosed epilepsy patients, (Persson et al., 2003). It was found that in fifteen patients undergoing ECG recordings, CBZ may suppress sympathetic and parasympathetic nervous system function. It was further acknowledged that in susceptible patients, the use of CBZ can result in increased patient vulnerability to cardio-respiratory effects as a result of autonomic system suppression and may therefore lead to premature death as a result of SUDEP. In other studies regarding the use of CBZ by epileptic patients it was shown that there was an
increase in sympathetic tone with subsequent increases in blood pressure and heart rate variability compared to healthy controls (Devinsky et al., 1994), another indication of increased SUDEP risk associated with AED use. In another study, Danielsson used a direct whole cell patch-clamp technique in order to establish the effects of another commonly used set of AEDs on the K⁺ rectifier channel (Kᵢᵣ) within the electrical conduction pathway of the heart, (Danielsson et al., 2003). Micro molar concentrations of both phenytoin and phenobarbitol were applied to cells containing the Kᵢᵣ channel until an IC50, a concentration when 50% of the receptor is blocked, was obtained. The potassium rectifier channel is particularly important when referring to the QT interval. Any kind of delay or disruption in this channel results in either the prolongation or shortening of the QT interval, with the potential to produce fatal arrhythmias and sudden death (Ackerman, 1998). Thus in any case related to SUDEP and the contributing risk factors, there must be some kind of exclusion criteria that takes into account the type of AEDs used by the subject.

**Preventative Measures in SUDEP**

Based on the proposed mechanisms of ANS disturbances associated with SUDEP, we chose to consider pharmacological measures used to control autonomic effects on cardiac function. A very well recognised group of medication that is used all over the world to control hypertension as well as prevent cardiac events associated with elevated adrenergic activity are β-adrenergic receptor antagonists otherwise described as β-blockers. As described previously, the autonomic nervous system exerts its effects through the sympathetic nerves, which innervate the heart through adrenergic β receptors. Activation of β adrenoreceptors, mainly β₁, results in increased chronotropic and ionotropic effects ultimately causing an increase in cardiac oxygen demand. The use of β adrenoreceptor antagonists in individuals
suffering from generalized seizures and increased adrenergic output may serve to prevent the development of tachycardia and other ECG abnormalities. As stated earlier, autonomic control of the heart originates in the higher brain centres, therefore it is possible to assume that a centrally acting target may be more effective compared to one that acts in the periphery. Clonidine has been shown to work directly at the central presynaptic noradrenergic (NA) receptors (Bolme & Fuxe, 1971). Clonidine is an α2-adrenergic receptor agonist, which exerts its effects at the level of the CNS resulting in hypotension (Guyenet, 1997). Specifically, both the α2A and α2C adrenergic receptor subtypes are responsible for presynaptic inhibition of noradrenalin release during CNS system processes such as the startle reflex, locomotion and the stress response (Kable et al., 2000). A number of studies have been conducted in the past in order to ascertain whether clonidine is capable of acting directly at the level of the heart. In isolated Langendorff perfused heart performed on rats (Salt, 1972) and guinea pigs (Csongrady, 1974), clonidine was shown to have a small positive ionotropic effect, however this was at high doses (0.1ml/mg) and direct application, therefore it is possible to assume that systemic doses would be too low to effect the heart directly. Further studies have shown a small population of α2 adrenoreceptors localized in the coronary arterioles (Saunders & Limbird, 1999), and vascular smooth muscle (Chotani et al., 2004), however no evidence was found to support clonidine action directly on the myocardium. Consequently, modulation of the presynaptic α2 adrenoreceptor in the CNS may serve to directly attenuate sympathetic output following seizure activity.
Aims and Hypothesis

This study hypothesized that seizurogenic activity evoked following systemic administration of an excitatory neurotransmitter KA is associated with disruption of adrenergic system function and conversely the development of cardiomyopathy.

Furthermore we hypothesized that this cardiomyopathy may be attenuated by the use of sympatholytic agents.

- Using an established model of seizurogenesis obtained following the administration of the excitatory glutamate receptor agonist KA, we examined the development of epileptiform seizures over a 48 hour time period.
- Based on the mode of action of KA, rats will receive subcutaneous injections of 10mg/kg KA previously shown to induce severe, level 5 seizures or equivalent tonic-clonic convulsions.
- In order to assess cardiac electrical activity during seizure development, rats will be implanted with EEG and ECG telemetric monitoring transmitters 7 days prior to induction of seizure activity.
- This study will correlate behavioral, cardiac and encephalographic activity during a range of seizure scores. The high level seizure activity is expected to have a greater effect on cardiovascular activity, resulting in significant cardiovascular disturbances. Different analyzing techniques and observational methods will be used in order to quantify the significance of cardiovascular effects during seizure activity.
- In order to discriminate a direct cardiotoxic effect resulting from a systemic administration of KA, this study employed a centrally acting α2 presynaptic
adrenoreceptor agonist (clonidine) to block sympathetic outflow from the CNS. The selective β1 adrenoreceptor antagonist (atenolol) was used to inhibit the effects of sympathetic neurotransmission directly on cardiac nodal activity.

- The effects of KA induced seizure induction in the presence and absence of sympatholytic pre-treatment will be assessed on ventricular morphology as additional evidence of cardiomyopathy.

- Based on the hypothesis suggesting the direct influence of seizure activity on disruption of cardiovascular function it is expected that adjunctive treatment with either atenolol or clonidine, will prevent the cardiovascular disturbances.
METHODS
**Materials**

All of the surgical equipment was provided by the Kerr and Sammut laboratories located in the Department of Pharmacology and Toxicology. KA, clonidine and atenolol were purchased from Saphire Bioscience PTY (New South Wales, Australia). The two-channel telemetric digital transmitters were obtained from Telemetry Research, Auckland, NZ, and were fully charged and sterilized in 2% glutaraldehyde solution prior to every surgery.

**Animal Care**

A total of 20 age-matched male Sprague-Dawley rats weighing 300-350 g were required for the entire experiment. The animals were obtained from the University of Otago Animal Resource Unit and were acclimatised in the Department of Pharmacology and Toxicology holding room for 7 days under controlled 12-hour light/dark conditions. Animals were fed *ad libitum* on a standard rat chow diet prior to experimentation. All surgical and euthanasia procedures were conducted in accordance with the University of Otago Animal Ethics Committee permit (ET03/10)

**Experimental Protocol**

The study animals were divided into four different treatment groups: saline control, KA seizure + untreated (drug vehicle only), KA seizure + atenolol and KA seizure + clonidine. Following acclimatisation, the rats were implanted with the surgical transmitters under general anaesthesia and allowed to recover for approximately 7 days prior to dosing with the drug vehicle, clonidine or atenolol as appropriate. Seizure induction was conducted using KA administration. Seizure scoring was recorded for a specified time period
following induction. Animals were terminated at the 48 hour point following seizure induction and the cardiac ventricles isolated and processed for ventricular morphological analysis.

**Animal Surgery (Electrode Placement)**

The rats were anaesthetised with ketamine hydrochloride 70mg/kg, domitor 0.3 mg/kg, and atropine 0.05 mg/kg. The animals were then placed on their backs to shave off the fur in the required regions (abdomen, neck and head). Body temperature was maintained throughout the surgery using a heating pad and rectal temperature probe. A two-channel digital transmitter was inserted within a subcutaneous pocket through a small incision in the skin overlaying the abdomen. One of the leads measuring cardiac activity was sutured onto the xiphoid process while the second lead was tunneled subcutaneously to the rostral thorax. The second ECG lead was then closely positioned next the right atrium by exposing the muscle covering the trachea and pushing the cardiac lead along the trachea into the anterior mediastinum. The remaining electrode pair were placed into a position immediately posterior to the foramen magnum so as to record the EEG signal. In order to achieve accurate EEG lead insertion into the cortex the rats were placed into a stereotaxic frame (KOPF, Narishige Scientific Instruments, Tokyo), where an incision was made through the skin lengthwise along the skull and then retracted. Using the accurate positioning of the stereotaxic frame’s attachable drill, three trephine bore holes were drilled 1mm (depth) into the skull with a size 0070 drill bit. These holes allowed for the insertion of two stainless steel anchoring screws, placed bilaterally into the skull using Bregma as the landmark, as well as allowing for electrode insertion, which was performed with extreme caution in order to avoid any further unnecessary damage to the surrounding brain tissue. The
stereotaxic coordinates for recording and reference electrodes were determined and placed as follows; recording electrode: 5.2mm posterior to bregma, 5mm left of middle, and 1mm in depth from the surface of the skull; reference electrode: 7mm posterior to bregma, 2mm right of middle, and 1mm in depth (see Fig 3.). In order to ensure that EEG recordings were not displaced during vigorous seizure activity, the electrodes were secured onto the surface of the skull using dental cement. Once the cranioplastic cement was dry, the electrode leads were further secured by carefully suturing each to the back of the neck, as previously described (Sawant et al.). The animals were then allowed to recover for approximately 7 days depending on the progress of recovery, with a three day follow up period of analgesic (captopril 1mg/kg), antibiotic (streptomycin, 0.1ml) and fluid (0.9% saline heated to body temperature, 10 ml), administered through subcutaneous injection (s.c.).
Figure 3: Schematic diagram representing surgical procedure of transmitter implantation. The transmitter was placed subcutaneously, at the level of the lower abdomen. The black ECG electrode was attached to the xyphoid process while the red recording lead was placed near the right atrium. Three holes were drilled into the skull, of which the green represents the recording electrode, the yellow the reference and the white depicts screw placement.
Animal Dosing

Atenolol
Following the surgical recovery period, the animals were divided into groups assigned for drug administration. The rats allocated to the atenolol group were given their first dose of atenolol three days prior to KA challenge, the day during, and then the two days following up until sacrifice on the second day following KA administration. Atenolol was delivered at 4mg/kg (oral, twice daily), as demonstrated (Belpaire et al., 1990), to be most pharmacokinetically effective route in the rat. Oral administration was achieved by producing small jelly capsules (gelatin + Gregg’s Raspberry mixture), containing atenolol adjusted to deliver the 4mg/kg dose based on each specific animal’s weight. This dosing was achieved by prior preparation of the atenolol-jelly delivery mixture at different strengths so as to deliver the same dose/weight ratio to each animal.

Based on the different weight classes provided (ranging from 250g to 400g animal weights), four different jelly mixtures were made, taking into account the amount of animals in the treatment group and thus the amount of individual doses required. This was calculated using a dosing calculation developed by a colleague (Mr Scott Smart) within the department.

Clonidine
The second animal treatment group received 100μg/kg of clonidine, (Zhang & Cheng, 2000), twice daily, three days prior to KA challenge, during, and the days following, up to the final day of sacrifice. The doses were delivered by s.c. administration and drug doses calculated based on the animal daily weight. Clonidine was injected in a total volume of 1ml of saline.
**Kainic Acid**

For seizure induction, all of the animals apart from those in the saline treated group, received 10mg/kg (in a 1 ml saline volume) subcutaneous injections of KA, immediately prior to undergoing behavioral analysis.

**Behavioral Analysis**

Following the recovery period, animals were weighed to establish accurate recovery, which included assessment of wound healing and weight loss that did not exceed 10% original animal weight. On the day of seizure induction, the animal was placed into a plexiglass observation chamber for an hour prior to the experiment in order to allow the animal to habituate to its environment. Following 30 minutes of recording the rat was removed from the observation chamber for injection and KA administered at 10mg/kg subcutaneously. The animal was then carefully placed back into the chamber. The respective behaviors were recorded, coded and logged onto a computer corresponding to the EEG and ECG trace recordings for 30 minutes baseline period followed by 3 hours of KA challenge, as described by Sawant *et al.*, (2009). The behaviors were recorded once every 15 seconds if they were being repeated and were then ranked according to a five-point scale that was previously defined in experiments performed by (Hesp *et al.*, 2007). These behavioral rating scores include hyperactivity (HYP), wet-dog shakes (WDS), head tremors (HT), circling (C), myoclonic jerks (MJ), mastication (M1), hind limb extension (EXT), scratching (SC), freezing (FRZ), rearing (REAR), salivation (SAL), and the highest level of tonic-clonic convulsions (CTC), an example of which is demonstrated in figure 4. The combined scores of the cumulative behaviors were then reported as the sum of the highest scores observed during each minute of observation.
over the entire length of the recording experiment. For example, even when a combination of different level seizure behaviors was present, the recorded seizure score would be that of the higher seizure behavior. Further hourly recordings were made at 6-7 hours, 24-25 hours and 48 hours following KA injection, during which the animals continued to be observed for any accompanying ECG abnormalities. Those animals that were in the drug treatment groups also continued to receive treatment for the entire 48 hours post seizure, until sacrifice, performed following the final period of recording. Animals treated with oral dose of atenolol were observed until jelly tablet was consumed following seizure periods to ensure accurate oral drug dose delivery.

**EEG and ECG Recordings**

EEG and ECG data was recorded using Maclab 4s signal conditioner and Chart 6 analysing software (ADInstruments, Sydney), as previously described (Sawant et al.,2009). Both EEG and ECG were acquired during the acclimatization period of 30 minutes prior and then throughout the specified time intervals mentioned previously. The ECG was sampled at 2000Hz, with receivers set to 0.1 Hz high pass and 1000 Hz low pass filters. Data recording was separated into specific recording periods over the entire 48 hours of KA induced seizures, this was also done for the control group. The recording time frames were set out as follows; first 30 minutes prior to KA administration for baseline measurements, full three hours directly following KA administration, followed by 1 hour of recording during the 6-7 hour, 23-24 hour and 47-48 hour post KA administration time point. The data was then analyzed in 1 minute blocks for all the recorded time periods. Additional studies analysed peri-ictal EEG and ECG activity in each group of animals during the 3 hour recording time frame.
Cardiac Isolation and Perfusion-Fixation

At sacrifice, rats were sedated using a bell jar saturated with halothane. Anaesthesia was maintained using halothane delivered via a nose cone. As soon as toe pinch reflexes were abolished in the subject animal, an incision was made in the body cavity in order to expose the superior vena cava. A 2ml blood sample was taken from the exposed superior vena cava using a heparinized 23G×1/4” needle and 5ml syringe and put on ice. One ml of 20mM KCl in normal (0.9%) saline was then administered into the vein, in order to immediately arrest the heart. The initial incision was then extended to produce a midline thoracotomy and the heart rapidly removed taking care to avoid any damage to the organ. The heart was then placed in ice-cold slush 0.9% saline solution and the aorta cannulated using a 16 gauge atraumatic needle and secured using a 0-0 suture, so as to allow normothermic Langendorff perfusion (73.6mmHg) with 0.9% saline containing 20mM KCl. Using small surgical scissors, the lungs, thymus and any other adherent non-cardiac tissue were removed in order to reduce perfusion resistance. Perfusion with the saline KCl solution was briefly maintained (5 min), so as to ensure that all blood constituents were washed clear of the coronary vasculature, and the heart was then perfused with a 10% neutral buffered saline (NBF) tissue fixation solution (50 ml). Once perfuse-fixed, the heart was bathed in NBF in a sample container so as to allow complete fixation and stored for 4 hours at 4°C. Hearts were then transferred into 70% ethanol and stored at 4°C prior to dehydrating and wax embedding.
**Histology**

The ventricular samples were sectioned along 4 transverse planes into 1.5 mm thick tissue blocks from apex to base and dehydrated using graded ethanol immersion and permeated using xylene prior to embedding in paraffin wax. Block sections were cut using a microtome (Leica Jung Autocut, Germany) at 4 µm thickness and mounted on glass slides for histological staining (Min *et al.*, 2003). The staining was performed using Erlich’s haemotoxylin and eosin stain (H & E), which stains the cell nucleus blue and the cellular contents pink. Sections were studied using light microscopy (Axioplan 3, Germany in association with Axiovision v.3.1 Image Analysis Software, Carl Zeiss Ltd, Germany) in order to identify ventricular morphological changes that may have occurred during the 48 hour period following seizure induction.

**Statistical Analysis**

Behavioral seizure scores were averaged per group at specific time points using Microsoft Excel software. All individual data representations were achieved through the use of Lab Chart Pro v. 6.0 and Histogram Analysis software. HR parameters were taken at 1 minute intervals and were averaged across all animal treatment groups, for specific time frames, using Microsoft Excel. Statistical analysis was performed using GraphPad Prism v. 5.0 software. One way ANOVA was performed on all parameters analysed with Bonferroni post-hoc tests used to compare treatment versus controls for each experiment. Results were expressed as mean ± standard error of the mean (SEM), with a *P* value of <0.05 considered to be significant.
RESULTS
**Morbidity and Mortality**

Throughout the experiment, a total of 2 animals, allocated to different groups had died. This was a direct result of anaesthetic overdose, where a combination of three different anaesthetic agents, variability in anaesthetic efficacy and lack of oxygen supply during surgery resulted in the subject animals succumbing to respiratory depression. All of the animals had experienced weight loss that was well below 10% of original body weight following the surgical procedure.

**Seizure Activity Following KA Administration**

Out of the 15 animals in the 10mg/kg KA treated group, all experienced a form of seizure activity that was equivalent to level 4 behavior or higher. There were a total of five animals that reached tonic-clonic convulsions, however, this was not specific in relation to any one particular treatment group. A total of 13 of the KA treated animals reached the highest level of seizure behavior in the first three hours following KA administration, while 2 out of 15 KA treated animals did not reach the highest level of seizure behavior until the 6-7 hour period of recording.
Figure 4: Representative examples of EEG recordings during seizure activity. Observed EEG abnormalities were found to occur at different stages of seizure following 10mg/kg injection of KA. Each trace is labeled concomitant with the specific behaviors that occur. Anti-clockwise from left; normal EEG with no seizure activity, initial stages of seizure level 1, freezing, higher level behaviors 3 and 4, tonic-clonic convulsions level 5, mastication occurs throughout.

Seizure Related Behavioral Observations

The averaged behavioral scores at each time point were significantly different to saline treated animals compared to KA treated groups ($P < 0.0001$). There was no significant difference between the atenolol treated animals and the KA-untreated group (figure 5). The averaged behavioral seizure scores were significantly lower in the clonidine treated group at $1.67 \pm 0.11$ compared to a mean behavioural seizure h score of $2.47 \pm 0.12$ in the KA-untreated group at the 2 hour post injection period ($P < 0.0001$). At the 6 hour recording period however, animals treated with clonidine had significantly higher averaged seizure scores compared to KA-untreated ($1.43 \pm 0.15$ vs. $0.73 \pm 0.09$, $P < 0.0001$).
Figure 6: Graphical representation of time to onset of initial level 4 seizure behavior in the atenolol and clonidine treated groups compared to KA-untreated.

Figure 5: Averaged sum of behavioral seizure scores for saline, KA-untreated, KA-atenolol and KA-clonidine treated animal groups. The x-axis represents time points following (“post”) 10mg/kg KA injection.
Clonidine pre-treatment appeared to delay the time to KA-induced high level seizure onset compared to KA-untreated, however this effect was not found to be statistically significant (figure 6). Similarly atenolol had no significant effect on the time to onset of level 4 seizures. Visual observations in relation to the type of seizure activity in the KA-untreated animal group did however, demonstrate ECG changes that resembled a type of cardiac abnormalities, particularly following the initial, 3 hour seizure period, and are depicted in figure 7.

![Figure 7: Three representative time points depicting EEG and ECG recordings at 15ms intervals in animal treated with KA. Baseline reading taken during the initial 30 minute period prior to KA administration, no ECG changes with average HR at 346 bpm. Ictal period (at time of seizure) readings taken one hour following KA administration, concomitant increase in HR to 412 bpm. Post-ictal (following seizure) recordings taken approximately 2 and a half hours following KA administration, EEG trace condensed and representative of prior seizure activity and concomitant ECG trace which is demonstrating disruption in wave amplitude with significantly elevated HR to 434 bpm.](image)

**ECG-derived Heart Rate Responses: Bradycardia**

Examination of HR responses across all drug treatment groups consistently showed the appearance of a bradycardic response occurring within 30 min of KA-induced seizure activity. HR was seen to decline to approximately 70% of baseline values in the KA-treatment group unlike the saline control animals (figure 8a). Compared to saline controls
(428 ± 6 bpm), KA-untreated animals exhibited a significant ($P < 0.0001$) decrease in HR of 283 ± 6 bpm, at the 30 minute period following seizure induction. HR in the atenolol-treated animals was recorded at 278 ± 5 bpm at the 30 minutes following KA injection, this value remained significantly $P < 0.0001$ different compared to saline-control animals responses (figure 8b). Compared to saline control animals, the clonidine treated group exhibited the largest reduction in HR during the 30 min post KA-administration period with heart rate averaging 228 ± 5 bpm. As well as being statistically significant ($P < 0.0001$), in comparison to saline-control, this HR response was also significantly ($P < 0.0001$) lower when compared to KA-untreated and KA-atenolol treated animals.

**ECG-Derived Heart Rate Responses: Tachycardia**

Following the initial period of bradycardia, HR increased significantly in the KA-untreated group, as demonstrated in figure 8. Compared to saline treated animals (390 ± 4 bpm), KA-untreated HR averaged 460 ± 2 bpm at the 2 hour time point following injection. This also occurred at the 3 hour time point where KA-untreated HR was significantly higher at 427 ± 2 bpm compared to saline animal, 374 ± 4 bpm, both measures were statistically significant with $P < 0.0001$. The increased HR was also observed to occur in conjunction with significant seizure activity (ictal) as well as more than 3hrs following seizure, as depicted by figure 7. Of the atenolol and clonidine treated groups, both groups appeared to delay the HR increase otherwise observed in KA-untreated animals. At the 1, 2 and 3 hour post injection periods, averaged HR values of the atenolol treated animals was significantly lower than KA-untreated, 322 ± 7 bpm vs. 365 ± 9 bpm, 410 ± 3 bpm vs. 460 ± 2 bpm and 384 ± 3 bpm vs. 427 ± 3 bpm, respectively, ($P < 0.0001$ for all three timer frames). Compared to KA-untreated,
clonidine treated animals had a considerably lower HR across all time periods. This was seen particularly at baseline, and at 2 hours post and 3 hours post KA injection, where the HR was at least 100bpm lower when compared to the KA untreated group, \((P < 0.0001)\). There were no significant differences between groups at the 6-7 and 23-24 hour recording period, however it was noted that two of the animals, one atenolol and one clonidine treated, suffered significant seizure activity in the 6-7 hour time frame.
Figure 8: Averaged HR of all treatment groups across all significant time frames. (A) Demonstrates HR of saline treated animals compared to KA-untreated, # = $P < 0.0001$. (B) Representation of drug treated groups atenolol and clonidine compared to KA-untreated, at significant time frames, * = $P < 0.0001$ compared to atenolol, # = $P < 0.0001$ compared to clonidine.
Individual Animal ECG Observations

Examination of ECG parameters during the initial bradycardic period revealed a subsequent absence and/or inversion of the P-wave as well as an increased incidence of skipped beats which gradually increased and then eventually subsided as heart rate began to recover back to baseline. Another observation during this period was the lethargic nature of the animals where they appeared to be sedated, exhibited by complete muscle relaxation and in some cases dragging of limbs, noted as discomfort behavior level 1. The incidence of skipped beats occurred across all treatment groups and was completely absent in saline treated animals (figure 9). However, the reported lethargy, occurring concomitantly with this period of bradycardia did not occur in the animals treated with clonidine.

Figure 9: Raw data from recorded ECG trace of an animal’s HR approximately 10 minutes following 10mg/kg injection of KA. (A) Represents baseline ECG prior to KA injection. (B) Skipped beats occurring every 2-3 beats, develops 10minutes following KA injection. (C) 20 minutes following KA injection, cardiac abnormalities, absence of QRS, evidence of AV block in bradycardic animal. (D) × 4 magnified view of skipped beats.
Out of the KA treated animals, 87% exhibited a disrupted P wave, 67% of which had an inverted P wave which lasted approximately 20 minutes from the time point of when the skipped beats began occurring (figure 10). Upon further observation of the ECG trace during periods of P wave inversion revealed the concomitant variation in QRS complex and ST amplitude. Compared to baseline, during periods of bradycardia and P wave changes, the QRS complex and ST segment increased in amplitude.
**Figure 10:** 1 minute averaged view examples relating to P wave disruption which occurred approximately 16 minutes following 10mg/kg injection. (A) Normal ECG wave from a saline treated animal. (B) Absent P wave, occurred consistently over a 20 minute period. (C) Inverted P wave, occurred consistently for approx. 20 minutes following KA injection.
Following the initial period of bradycardia, all three animal groups which received KA experienced significant seizure activity which was directly associated with ECG abnormalities. Compared to saline-controls, KA treated animals were measured to have increases in HR during periods of significant seizure activity. The HR increases ranged in magnitude of up to 100 bpm increases in rate, which occurred ictally but did not progress beyond the seizure event. Furthermore, seizure events appeared to produce amplitude variations within the ECG trace that were not associated in HR changes (figure 8). Significant increases in HR were also evident during post-ictal periods and occurred without any observable EEG trace variations associated with seizure activity.

![Figure 11](image)

**Figure 11:** Raw data from KA-untreated animal comparing HR variation at baseline to periods during seizure events (ictal) and following seizure activity (post-ictal).

**Heart Rate Parameter Observations**

As expected the RR interval findings were inversely correlated to HR. Compared to saline, KA-untreated animals had a 30% increase in the RR interval at the 30 minute time period, statistically significant with $P < 0.0001$. At the 2 and 3 hr post averaged periods,
RR interval was significantly lower in the KA-untreated animal group compared to saline. RR interval changes that were observed in the KA-untreated group were also evident in the atenolol-treated animals which were not significantly different across all time points. The RR interval further demonstrates the consistency with the findings regarding alterations in cardiac rhythm following KA administration across all treatment groups.

![Graph showing averaged RR interval values across specific time points, compared between treatment groups. * = statistically significant compared to KA-untreated, P < 0.0001, # = statistically significant compared to saline, P < 0.0001.]

**Figure 12:** Averaged RR interval values across specific time points, compared between treatment groups. * = statistically significant compared to KA-untreated, $P < 0.0001$, # = statistically significant compared to saline, $P < 0.0001$.

It was already noted that the P wave was significantly affected following KA administration as demonstrated in figure 10, it must also be noted that PR interval was also altered. At the 30 minute and 1 hour post injection time frames, both KA-untreated
and atenolol treated animal groups had significantly elevated PR interval lengths of 50 ± 0.7ms and 50 ± 0.6ms for KA-untreated, 50 ms ± 0.4 ms and 50 ± 0.4 ms for atenolol compared to saline group with PR lengths of 40 ± 0.4 ms and 40 ± 0.4 ms, respectively, \( P < 0.0001 \). Clonidine, however, was observed to produce a similar trend to saline treated animals and was significantly lower compared to KA-untreated at both time frames, 30 minutes and 1 hour post, where the biggest effects on \( P < 0.0001 \). Analysis of the adjusted QT interval (QTc), revealed significant QT lengthening in the KA-untreated group compared to saline, at all three time frames following KA injection, corresponding to beginning of seizure activity, \( (P < 0.0001) \). At 1 hour post QT was shortened to 0.041 ± 0.001 s, 0.0048 ± 0.001 s at 2 hours post and 0.0046 ± 0.001 s at the 3 hour time frame compared to saline QT length of 0.031± 0.002 s, 0.038 ± 0.001 s and 0.038 ± 0.001 s, respectively (figure 13a). Although atenolol treated animals had a significantly elevated QTc length at baseline compared to KA-untreated and saline \( (P < 0.0001) \), there was no further significant difference between this group and KA-untreated animals. Clonidine treated animals on the other hand showed a trend that was in conjunction with saline treated animals throughout the first 3 hours of the recorded experiment. Compared to KA-untreated, clonidine treated animals had a normal QTc length at the 1, 2 and 3 hour time frames, which were not statistically different to saline-controls, however at least 25% shorter than KA-untreated and atenolol treated animals with \( P < 0.0001 \), (figure 13b).
Figure 13: QTc lengths averaged across significant time frames for Saline, KA-untreated, KA-atenolol and KA-clonidine. (A) Saline compared to KA-untreated, ** = $P < 0.0001$, * = $P < 0.001$. (B) KA-untreated compared to atenolol and clonidine, ** = $P < 0.0001$ statistically significant compared to KA-untreated, * = $P < 0.001$ statistically significant compared to KA-untreated.
Individual ECG Observations (Ictal Period)

Out of the 15 KA treated animals, 6 (40%), experienced tonic-clonic convulsions, approximately 2 from each treatment group. Following consistent observation of individual animal recordings at time of tonic-clonic convulsions it was evident that there were considerable changes in HR during this high-level seizure period. At least a 25% increase in HR was observed directly prior to tonic-clonic activity and lasted the entire duration of the convulsions as well as a further 10 seconds following. One of the animals from the atenolol-treated group experienced periods of tonic-clonic convulsion 12 times over a 1 hour time-frame. Each period was accompanied by the HR alterations consistent with increases from 400bpm to 500bpm lasting no longer than 30 seconds, with concomitant increase in ECG amplitude. As well as alterations in HR during level 5 behaviors, HR changes were also noted to occur in conjunction with level 4 behaviors such as salivation and foaming (Figure 14). These ictal changes included drastic increases and decreases in HR of up to 50%, amplitude variation, and incidence of skipped beats like that observed during the initial period of bradycardia that occurs directly following KA administration. These trends are demonstrated further in figures 15, 16, 17 and 18. In comparison to saline treated animals (figures 15a and b), the KA-untreated animal depicted in figures 16a and b are shown to experience an initial decrease in HR, followed by a significant increase in HR as well as consistent HR variation which correlates to high-level seizure behaviours that occur within the initial 3 hours post KA administration. Similar trends are observed in animals treated with atenolol (figures 17a and b) and clonidine (figures 18a and b).
**Figure 14:** HR abnormalities which occurred over the first 3 hour recording period in animal #9 (atenolol-treated). First row demonstrates the EEG trace during which the animal experienced a combination of level four seizure behaviors. Below are the concomitant ECG traces that are representative of the abnormalities that occurred during periods of seizure activity. Column (A) demonstrates the variation in HR amplitude and the associated HR variations that occur during that particular period of EEG activity. (B) depicts an example of bradycardia and skipped beats that occurred throughout the final hour of the 3 hour recording period. The ECG abnormality occurred in conjunction with foaming, (level 4 seizure behavior).
Figure 15: Individual data for saline-control rat, demonstrating events that occurred over the initial 3 hours of recording. (A) Represents the raw data of HR, taken at 2 minute intervals, correlated to seizure spike frequency. (B) is representative of EEG trace and the concomitant line graph of the behavioral score.
Figure 16: Visual representation of KA-untreated rat during the initial 3 hour recording period. (A) HR taken at 2 min intervals, correlated with seizure spike frequency. (B) Raw EEG trace versus line plot of behavioral scores. Both graphs demonstrate a clear trend where seizure activity appears to be suppressed in the initial 30 minutes following KA administrations. This is then followed by a significant increase in seizure activity, concomitant with elevation in HR and behavioral scores.
Figure 17: Visual representation of atenolol-treated rat following KA-administration in the initial 3 hours of recording period. (A) HR changes correlating to seizure spike frequency. (B) Raw EEG trace and superimposed behavioral score represented as line graph.
Figure 18: Visual representation comparing HR to seizure frequency and raw EEG amplitude to seizure score in rat treated with clonidine. (A) HR compared to seizure spike frequency. (B) Raw EEG trace and superimposed line graph of behavioral scores that occurred in the first 3 hours following KA administration.
Figure 19: Histopathological changes in apex and level 2 of heart tissue obtained from saline-control, KA-untreated, atenolol-treated and clonidine-treated rats at 48 hours following KA insult, visualized using H & E stain. (A) & (E) saline-control rats, tissue appearance is tightly packed cardiomyocytes, with no visualization of cardiac damage. (B) KA-untreated rat with evidence of tearing of myofibres associated with myocardial necrosis. Arrows indicate non-specific inflammatory cell infiltration. (C) atenolol-treated rat with evidence of reversible ischaemia along with major oedema. (D) & (H) clonidine-treated animals appear to have no signs of cardiomyocyte damage. (F) KA-untreated rat showing further evidence of major oedema and nuclear vacuolization. (G) atenolol-treated rat with evidence of oedema and nuclear vacuolization. (I) magnified image of slice image (G), demonstrating presence of a polymorph indicative of inflammatory cell infiltration.
Histology

The ventricular cardiac sections recovered for H & E staining were all obtained consistently without any significant ischaemic interval. The cardiac tissue was isolated from animals who had experienced level 4 seizure behavior or higher. Examination of tissue sections taken at 48 hours post KA from the ventricular apex and level 2 (1.5-3mm from apex) layers of KA treated animals that had undergone considerable seizure activity following KA administration, showed evidence of significant oedema as well as nuclear vacuolization (figure 19b & f). Along with these early signs of reversible ischaemic cardiac injury, the atenolol-treated animal (figure 19g & i) also showed evidence of non specific inflammatory cell infiltration, indicative of necrosis and irreversible cardiac damage. On the other hand, tissue sections taken from clonidine-treated animals bore no evidence of cardiac damage compared to KA-untreated and KA-atenolol treated animals. Cardiac tissue had no visible oedema nor was there any evidence of ischaemic injury in the clonidine-pretreated animals at 48 hours after seizure induction.
DISCUSSION
KA and Seizures
As it was proposed, 10mg/kg subcutaneous injections of KA produced significant seizure activity that was at least equivalent of level 4 seizure behavior or higher across all animal groups. Evidence of the KA-induced seizure activity was observed 30 minutes following KA administration. During the first 30 minutes following KA administration however, the EEG trace was lower in amplitude compared to baseline and saline treated groups, and was accompanied by significant bradycardia. In 87% (13) of the animals that were administered KA, recordable EEG and observable behavioral activity was present for the first 3 hour recording period. 13% of the treated animals displayed seizure behavior for the next period of recording at 6-7 hours following initial KA injection however, across all groups, no evidence of seizure activity was seen at 24 and 48 hours post recording periods. Pharmacological treatment with atenolol or clonidine did not significantly alter seizure levels outcome, clonidine did however appear to increase time to high level seizure onset. Based on the known mechanism of action of clonidine, this finding of potential seizure reduction is controversial as there are conflicting thoughts concerning the direct effects of clonidine in respect to seizure activity. Given that clonidine is an α2 receptor agonist, numerous studies have suggested that clonidine serves to have an anxiolytic effect, thus potentially preventing or reducing seizure activity (Hidalgo et al., 2005). Conversely, other studies (S'derpalm & Engel, 1988) demonstrated a biphasic effect dependent on clonidine dosage following IP administration in rats. Clonidine was used to establish its anxiolytic affects using two anxiety-related behavioral tests: a modified Vogel's drinking conflict model and Montgomery's elevated plus-maze. Evaluation of both models demonstrated that low dose clonidine (10 µg/kg) produced
anxyolitic-like effects, while higher doses (80µg/kg) produced anxyogenic-like effects. S’derpalm & Engel reasoned that this was due to the potential for high clonidine concentrations to modulate both α1- and α2- adrenoreceptors (S’derpalm & Engel 1988). Previous studies developed in order to explore the potential for this biphasic effect to occur used microinjections of clonidine on single neurons in the somatosensory cortex of the rat. Bradshaw et al., (1982), showed that clonidine may be acting as a partial agonist at excitatory α1-adrenoreceptors on cortical neurons.

In addition, there are also other considerations that need to be explored. As mentioned previously there was a period of reduced EEG and ECG activity in the first 30 minutes following KA injection which was concomitant with reduced HR. In an attempt to explain this phenomenon it is proposed that this bradycardic effect is due to afferent stimulation of the vagus nerve and higher brain regions such as the LC. The LC has long been recognised as the main source of NA in the brain as its projections innervate most of the largest recognised centres such as the cerebral cortex, thalamus, cerebellum, midbrain and spinal cord (Aston-Jones et al., 2009). Therefore it is interesting to note that in the presence of the preganglionic NA release inhibitor, clonidine, there was no apparent increase in seizure activity compared to the other treatment groups in our study; instead our data showed that clonidine administration resulted in a delay in seizure onset.

**KA induced Bradycardia**

All of the 15 animals that received KA injections (10mg/kg), experienced considerable reduction in heart rate that lasted for approximately 30 minutes with a constant occurrence of skipped beats that progressively intensified over time until the heart
recovered, prior to ictal period. The concomitant absence of any kind of encephalographic spiking during this time suggests the involvement of the ANS may be activated both within the CNS as well as at the periphery. This parasympathetic cardiovascular response has been previously reported by only one other study, where Ohta et al., (1991) observed the depressor response in a KA treated animal, following an initial period of tachycardia, which was reasoned to be attributed to cardio-vagal stimulation but not described further. The study however only observed cardiovascular responses to KA with no assessment of brain wave activity.

The sympathetic nerve contains preganglionic as well as many postganglionic projections that interact with the periphery thus making the sympathetic system one of the central targets for cardiovascular modulation following KA administration. Although both pre- and post ganglionic projections have been demonstrated to contain structural glutamate receptor analogues (Gill et al., 2000), as have the cells specific to conducting systems within the heart (Mueller et al., 2003), there is not enough evidence to support the hypothesis that these receptor analogues have a functional response to exitotoxins. The major consistent finding that supports the existence of peripheral glutamate receptor types is the indication of cardiovascular pathology observed across a number of different mammalian species following both domoic and kainic acid exposure (Hinoi et al., 2004; Gill et al., 2007). It is arguable whether the cardiac damage is due to a direct peripheral receptor binding or a result of seizure activity produced by the KA at the level of the CNS that disrupts the cardiac control centres (Vranyac-Tramoundanas 2011).

Based on the current findings it is possible that systemic KA produces its initial cardiac action through the vagus and thus the parasympathetic system. Currently, there is a large
amount of literature that supports the idea of vagal stimulation to reduce seizure in both human and animal models. Ben-Menachem and co-workers (Ben-Menachem et al., 1999), assessed the long-term effects of vagus nerve stimulation (VNS) in 64 patients suffering from refractory epilepsy. Observations that VNS stimulation resulted in a reduction (>50%) in the occurrence of seizures led the authors to propose VNS as an effective treatment. Other researchers used electrical vagal stimulation in the rat in order to attempt to reduce secondarily generalized seizures induced by pentylenetetrazol. McLachlan, (1993), found that during 20s vagal stimulation, the inter-ictal spike frequency was reduced by 33% while the residual spike amplitude was similarly decreased. Similar reductions in EEG spike frequency were observed during the initial 30 minute bradycardic period in our experimental animals.

The vagus nerve carries afferent information from the higher tissues such as those of the neck and head but also the abdomen, while parasympathetic efferents extend to the heart, lungs and major gastrointestinal tract (Ramani, 2008). Afferent fibres from both the soma and viscera form 80% of the vagus nerve. The proposed mechanism associated with VNS mediated reduction of seizure activity is concentrated around the influence on the nucleus of the solitary tract (NTS) and the higher brain centres that project further. Thus mainly involving the reticular activating system where vagal stimulus is transmitted through projections extending from the NTS to the reticular formation and thence stimulating diffuse projections to the cortex and thalamus, in particular, the ascending projections to the parabrachial nucleus (PB) and LC. The LC was shown to form the primary centre for NA release (Krahl et al., 1998). Stimulation of the vagus nerve and subsequent activation of LC resulting in NA release is proposed to produce a reduction in seizure activity, as
determined by lesion studies. An attempt to demonstrate this effect is depicted in figure 20.

Based on the volume of literature supporting the role of afferent VNS on the reduction of seizure activity, we suggest that the reduction in encephalographic activity observed in the initial 30 minutes following systemic KA administration in our study results from KA-mediated effect on the vagus nerve. Due to subsequent reduction in HR to approximately 40% of baseline in the 30 minute post–KA administration period, we also propose that this vagal action not only projects afferently to the brain but also in an efferent manner towards the heart. This potential dual action that activates both peripheral and central effectors would require a centrally mediated effect at the level of the brainstem, where the nodose ganglion is known to contain both GABAergic and glutamatergic terminals. This action was demonstrated by (Travagli et al., 1991), who reported that following vagal and perivagal stimulation they were able to successfully evoke both excitatory and inhibitory spontaneous and synaptic currents in the dorsal motor nucleus of the vagus. Further whole-cell voltage clamp recordings of synaptic currents and adjacent pharmacological intervention demonstrated that the inhibitory synaptic currents are mediated by GABA activated Cl⁻ channels, while excitatory currents are mediated by ionotropic glutamate receptors of the NMDA and non-NMDA glutamate receptor subtypes. These findings further imply that KA has the potential to exude its effects in both directions along the vagus as the spinal vagal nucleus would appear to accommodate KA binding.

With this dual mode of action in mind however, studies which have used vagus nerve stimulation in order to establish its anti-epileptic qualities have found no concomitant
adverse reactions that concerned the efferent vagal pathways (Ben-Menachem, 2001). Although this idea regarding VNS does disregard the proposed theory it must also be acknowledged that VNS is performed electrically, where stimulation could be maintained mainly in an afferent direction. When considering the application of chemical excitatory compounds such as KA, we cannot simply rule out the binding profiles as well as specificity of receptor activation, which is difficult to define in a living animal model.

Consideration should also be given to the suggestion that KA may initially be acting directly at brain regions involved in cardiac control in such a way as to produce bradycardia. The amygdaloid complex is just one of the brain regions central to the autonomic regulation of conditioned behavioral responses. Goodman et al., (1999), found that following amygdaloid kindled seizures in the rat there was an elevation of blood pressure, which was directly followed by a significant reduction in HR. In order to examine this autonomic response, these researchers applied atropine to produce a muscarinic receptor blockade and abolished the seizure-induced bradycardia. These findings further imply that depending on the origin, seizure activity may initially result in parasympathetic stimulation. This finding ultimately acknowledges the potential for the proposed theory suggesting bidirectional modulation of the ANS by KA.

In future experiments, the proposed action of KA on ANS modulation could be fully explored in a vagotamised rat model. Another way that may establish the excitotoxic effects on the vagus nerve would be to repeat the study in rats pretreated with a parasympathetic nervous system antagonist such as atropine. A previous study by Ohta et al., (1991), discussed earlier where bradycardia was also noted following systemic KA, showed that this cardiac depressor effect was abolished with the administration of methyl
atropine. A lack of understanding in regards to this effect resulted in Ohta and colleagues to reason that the depressor response may be attributed to the specific strain of rat (Wistar-Kyoto) used throughout the experiment, this idea was however never followed up.
Figure 20: Schematic diagram demonstrating the potential action of systemic KA injection. Red arrows are associated with the afferent action that exudes its action at the brain stem and higher brain regions in the first 30 minutes following 10 mg/kg KA injection. The blue arrows depict the efferent pathway that may be activated following KA injection that results in bradycardia. The purple area represents the centre for KA binding and activation in this initial period of activity. LC = locus coeruleus, RF = reticular formation, NTS = nucleus of the solitary tract.
Tachycardia Following KA

As expected, the animal group that had received 10mg/kg KA alone, had considerably elevated HR compared to saline treated animals at the 2 and 3 hour time frames post injection, where seizure activity was at its highest. Based on the exitotoxic nature of KA, which may initially stimulate discreet parts of the brain and then spread electrical seizure activity throughout all regions and both hemispheres, it is possible to reason that the numerous ANS control centres would be affected. In this study, there is evidence of HR increases of 10% during ictal periods, as well as constant HR variations that show irregularity in cardiac beats throughout the entire, primary 3 hour recording period. Similar findings are reported to occur in human patients, which validates the significance of the current results and their implication regarding epilepsy and cardiac abnormalities. Zijlmans et al., (2002), retrospectively analysed 281 seizures and found that in 91% of patients there was at least a 10bpm increase in HR around seizure onset time period. In 49% of patients the increase in HR preceded seizure onset and duration and was accompanied by ECG disruption consistent with conduction and repolarization abnormalities. Comparatively the animals used in this study showed HR increases of at least 50bpm at the 2 hour post KA administration period.

The heart may be influenced in two important ways as the body experiences stress, such as that resulting from behavioral disturbances in response to seizure activity. One of these responses may be through indirect action on the adrenal medulla and release of adrenaline onto cardiac adrenoreceptors, thereby increasing HR and cardiac workload (Harvey et al., 1984). Direct sympathetic innervations of cardiac muscle through exaggerated ANS projections following seizure activity, may also result in positive
chronotropic effect. Consequently, atenolol and clonidine were used in this study to establish the mechanism and origin (peripheral or central) of the ensuing cardiac injury. KA alone caused significant tachycardic effects at (2 and 3 hours post administration). These effects were attenuated by pre-administration of both atenolol and clonidine resulting in the reduction of HR to baseline levels. Clonidine pretreatment in KA dosed animals resulted in consistently lower HRs below those recorded in the saline-control animals which may propose that clonidine is a more effective cardio-protective treatment in a KA-induced seizure model compared to atenolol. These findings may also be solely attributed to method of drug delivery where clonidine was administered through systemic injection, while atenolol was administered orally. Oral drug delivery is subject to first pass metabolism which in the case of atenolol, could have resulted in a low bioavailability, below the effective therapeutic dose. This protective effect produced by clonidine also suggests that the influence on the heart may occur as a direct interaction on the cardiac control centres, thus allowing clonidine, a centrally acting α2 receptor agonist (Guyenet, 1997), to directly prevent cardiac damage caused by large quantities of NA released through the ANS, as the proposed mechanism of cardiac damage in this animal model.

**HR Parameter Changes**

Animals treated with KA alone had experienced skipped beats either during bradycardia or ictally, they also had significantly altered P wave amplitude, PR lengths and QTc intervals compared to the saline treated group. As it is now generally accepted that HR parameters may be significantly influenced by seizure activity (Brugada et al., (2004); Whitsel et al., (2001); Surges et al., (2004) and Gaita et al., (2003), therefore the
presentation of ECG changes following seizure induction in this rat model is consistent with the literature. Primarily however, there is minimal evidence supporting the occurrence of P wave and PR length disruption associated with a reduction in HR like the one seen in this study. Although skipped beats are often considered to be benign in most cases, in 50% of patients they are actually of cardiac origin, (Weber & Kapoor, 1996), and can be symptomatic of life-threatening arrhythmias. According to clinical assessment of cases brought forward by physicians, Abbott (2005), describes palpitations or ‘skipped beats’ as having life threatening implications, especially when they occur in conjunction with dizziness, near-syncope or syncope, the condition which consistently accompanies skipped beats that are observed in our study, in the initial 30 minutes following KA administration (denoted as anaesthetic-like behavior).

P wave amplitude was disrupted in 87% (13) of animals that had received KA administration, irrespective of pretreatment. In 67% (10) of the 15 total animals that received KA there was a complete inversion of the P wave during the first 30 minute post administration period. There is a substantial lack of scientific literature regarding P wave amplitude and its clinical implication in cardiac function. However, this is clearly a significant cardiac parameter that is altered substantially in the current experiment. P wave morphology and specific pacing techniques are used extensively in determining the origin and factors associated with initiation of atrial fibrillation (Yamane et al., 2001; MacLean et al., 1975; Tang et al., 1995). Through the use of 12-lead ECG’s, Yamane and colleagues studied the P wave during sinus rhythm and pacing at six different sites. Depending on the location of electrode placement they were able to obtain ECG traces in which the P wave appeared either positive, isometric or negative. Therefore, based on the
current understanding of P wave morphology and findings that have been demonstrated in the current study, it could be possible to assume that the inverted P wave that occurs during periods of bradycardia could be a result of a change in sinus rhythm and ectopic focus (Farina et al., 2009). It is possible to reason that the SA node is no longer the primary pacemaker due to ischaemic or pharmacological influences on the cardiac electrical conduction system following KA administration, thus producing P wave inversion as a result of pacemaker potentials originating closer to the AV node. This potential change in set pacemaker potential may result in inadequate transmission of contractile stimuli from the atrium to the ventricles, thus resulting in compromised cardiac function (lack of QRS peak). Upon further observation of the individual ECG trace obtained during periods of bradycardia in the current study (figure 10), revealed other important parameter changes that require mentioning. Along with P wave inversion, figure 10c demonstrates a concomitant increase in QR amplitude as well as ST segment elevation. These changes are indicative of sinus bradycardia that is commonly reported in patients undergoing observations using 12-lead ECG recordings (Hampton, 2008). However, upon further averaging of P wave and QR amplitude as well as the ST segment length and comparing baseline values to the period of bradycardia, no significant changes were found in the QR amplitude and ST segment length compared to the changes observed in P wave amplitude. It is difficult to interpret the changes occurring during the period of bradycardia, this is made particularly difficult based on the limitations involved with using a 2-lead system for recording.

Assumptions may be made in regards to the significance of the observable changes in P wave amplitude, however it is far more appropriate to focus on parameters that are
clearly established in their representation and possible implication when assessing cardiac abnormalities, such as PR length. During the first hour recording period following KA administration, the PR interval was significantly longer in the KA-untreated animal group compared to saline-controls. The PR duration is representative of the conduction time from the sinus node through to the atrium, AV node, Purkinje fibres and the ventricular myocardium. A delay in the PR interval represents the occurrence of a first-degree atrioventricular block that is not necessarily indicative of bradycardia but may potentially arise from sinus node dysfunction (Mangrum & DiMarco, 2000). A second-degree atrioventricular block is more common during bradycardia and is defined in humans as a block that occurs when rhythmic firing of the atrium fails to conduct to the ventricle at a ratio of 1:1. Based on the observations of the bradycardia occurring across all treated animal groups following KA administration suggests that KA is in fact producing some kind of cardiac damage in the first degree (figure 21). The failed conduction ratio of atrium to ventricles of 1:1 occurred in animals which experienced severe tonic-clonic convulsions, suggesting a correlation with the ventricular morphological damage obtained in this study. As reported by Mangrum & DiMarco, (2000), clinical management is required for any patient experiencing this type of bradycardia with potential AV block. Regular ECG monitoring and further testing using invasive electrophysiologic techniques is recommended in order to eliminate the risk of fatal-arrhythmia. Thus the significant increases in PR length that are seen in KA treated animals, especially at the 1 hour post administration time point where HR has recovered back to baseline values, suggests that seizure induced PR interval variations may contribute to SUDEP. Interestingly enough, when observing the effects of the atenolol
and clonidine treatment groups compared to KA-untreated, atenolol had no effect in reducing the prolongation of the PR interval. However, clonidine treated animals had significantly lower PR interval lengths that were equivalent to saline-control animal levels at the 30 minute and 1 hour post administration time points. This further implicates the involvement of direct CNS to ANS activation during seizure activity which is altered by clonidine but not peripherally acting atenolol, thus suggesting clonidine to be a more effective preventative treatment for cardiovascular disturbances that occur in response to seizure.

*Figure 21:* Example of bradycardia and skipped beats, possible depiction of sinus bradycardia with premature atrial contraction.

Another parameter that was significantly altered was the adjusted QT interval or the QTc. The QTc interval was significantly longer in the KA-untreated animal group compared to saline-control animals for up to 3 hours following KA administration. This prolongation occurred in conjunction with increasing seizure activity. The QT interval is mainly a representation of the duration of the complete depolarisation and repolarization cycle of the ventricular myocardium. This suggests that seizure activity also produces cardiovascular disturbances associated with ventricular contraction, otherwise termed the
QT interval. Variations in the QT interval that ultimately result in prolongation may predispose and are indicative of numerous cardiovascular disorders associated with cardiac morbidity, (Whitsel et al., 2001; Zabel et al., 1998; Brugada et al., 2004). One of the most highly recognised conditions associated with QT interval elongation is known as the long QT syndrome. Long QT is a serious condition and is associated with the precipitation of torsade de pointes ventricular tachycardia which may ultimately result in sudden death (Khan, 2002).

As with all other cardiac parameters that were assessed in this study, atenolol treatment failed to prevent QTc elongation in KA-untreated animals. Furthermore, the atenolol treated group had the same or higher QTc interval compared to KA-untreated. Conversely, clonidine treated animals appeared to be completely protected from KA induced QTc elongation at all three significant time points (1, 2 and 3 hours post KA administration). This observation further reinforces that the cardiovascular abnormalities are most probably originating from the CNS, rendering atenolol completely ineffective as this drug treatment does not cross the blood brain barrier, acting in the periphery where atenolol fails to prevent cardiac damage and arrhythmias that occur as a result of the lock-step phenomenon.

**Histological Observations**

At 48 hours following KA administration, there is clear evidence that KA produces cardiac damage. All KA-untreated animals had prominent micrographical evidence of oedema and myofibril tearing as well as nuclear vacuolization (indicative of early stages of reversible cardiac ischaemia) and non-specific inflammatory cell infiltration. Atenolol
treatment failed to prevent these morphological changes arising in the ventricular myocardium. This correlated with the lack of significant effect of atenolol on the ECG changes reported. Ventricular myocardium from the atenolol treated group demonstrated equal amounts of oedema and vacuolization as well as the presence of polymorph infiltration (figure 19i). A polymorph or polymorphonuclear leukocyte is a type of white blood cell that is part of the innate immune response and is not only representative of primary, reversible cardiac cell damage, but also plays a critical role in pathogenesis of cardiac tissue (Afanasyeva, et al., 2004). As mentioned previously, there is no reason to believe that systemically injected KA has any direct influence on the heart as existing literature fails to demonstrate any substantial evidence regarding fully formed and/or functioning glutamate receptors on myocardial cells or within the periphery (Gill et al., 2000; Mueller et al., 2003). Therefore, we can assume that the cardiac damage currently seen is due to considerable seizure activity and subsequent CNS and ANS stimulation of cardiac centers. This assumption that is associated with cardiac damage occurring as a result of the lock-step phenomenon is further implied by the results regarding treatment with clonidine. The clonidine treated animal hearts showed no evidence of damage in comparison to KA-untreated and atenolol treated animals. Ventricular myocardium appeared equally viable morphologically as that seen in the saline-control animals. The clonidine findings as well as the known action of clonidine in regards to suppression of the SNS, further suggest cardiac damage to be attributed to a sympathetic storm arising from seizure activity. It has been demonstrated that the heart eventually succumbs to ventricular hypertrophy as a result of NA spillover during sympathetic storm (Barton et al., 2007). It is possible to assume that this NA increase is the mechanism by which the
heart develops early stages of ischaemic cardiac damage such as the oedema that is seen in KA-treated animals. The ischaemic damage is evident of catecholamine toxicity that has also been described by our colleagues (Vranyac-Tramoundanas et al., 2011). This micrographical evidence of damage appears to be confined to the subendocardial region of the ventricular myocardium, which suggests that due to ongoing arrhythmias that occur throughout the initial seizure period, the surrounding blood vessels vasoconstrict as a result of rigor, resulting in reduced blood to the subendocardial region (Westerhof et al., 2010). Clonidine may be acting to protect the heart from damage by inhibiting NA spillover onto myocardial cells which results in the primary stages following significant seizure activity (first 48 hours).

Based on the current histological findings, as well as the current hypothesis suggesting that SUDEP arises through a pathological myocardial response to ANS stimulation, it is possible to propose two main mechanisms of action that may predispose the individual to premature death. The primary or acute mechanism to explain this interaction is the lock-step phenomenon proposed by Stauffer and colleagues where seizure activity directly stimulates the ANS in such a way as to produce significant changes in heart rate variability during or directly following the ictal period. This can provoke instantaneous or acute responses resulting in potentially fatal arrhythmias such as described in the status epilepticus animals. It is also important to consider the secondary or chronic consequences generated by ongoing periods of seizure activity in an individual that suffers from epilepsy over a prolonged period. Here, the progressive increases in ANS output resulting in compromised blood flow to sub-endocardial tissue results in a reduction in cellular integrity, and compromised ventricular function, as that suggested
by the early signs of ischaemic myocardial damage observed in histological slices (figure 19). Long-term evidence of this effect has been shown to include perivascular and interstitial cardiac fibrosis in rats exposed to domoic acid (Vranyac-Tramoundanas et al., 2011). In the latter case the subject may still end up suffering from SUDEP however, this would occur as a result of long-term cardiac damage that would eventually result in compromised cardiac function and remodelling, potentially leading to a heart attack. These mechanisms are summarized in the following figure 22.
**Figure 22**: Two mechanisms describing the potential cardiac effects as a result of KA administration. Step 1; represents damage involving the lock-step phenomenon, this is the example of acute damage that results due to an electrical malfunction and subsequent cessation of heart beat/torsade de pointes/ventricular arrhythmia leading to sudden death. Step 2; demonstrates the proposed chronic mechanism involving progressive damage to myocardial cells which eventually leads to reduced cell viability and potential heart failure.
Limitations to the Experiment

Although KA is a neurotoxin and is a valid model of seizure induction resembling epilepsy, subcutaneous delivery of KA may not provide a response limited to the CNS. The exact receptor binding profiles of KA to peripheral and cardiac receptors is not well defined, and therefore presents uncertainty as to whether seizure alone is what directly responsible for the cardiomyopathy described and not the effects of systemic KA. This theory has however been strongly argued against in a recent publication by our group comparing intrahippocampal (i.h.) and intraperitoneal (i.p.) domoic acid delivery. In future experiments the conflicting factors could be avoided in a number of ways: localized i.h. KA administration will be used to prevent the possibility of a systemic release of KA on the heart. The presentation of bradycardia observed in the first 30 minutes of the experiment should also be examined. While atropine could be used to inhibit KA-induced vagal responses, a surgical vagotomy performed prior to KA administration would serve to deliver an answer in the absence of atropine induced tachycardia. Similarly the results can be repeated in a cardiac sympathectomy model to determine the evidence of sympathetic involvement in the ensuing cardiomyopathy. The use of a peripherally acting glutamate receptor antagonist could also be applied in order to determine whether KA is acting peripherally on cardiac receptors. Initially the current proposal was intended to maintain the animals for 14 days following KA administration however, based on the complications of transmitter surgery which occasionally resulted in tissue necrosis around the implanted transmitter site, animals were sacrificed at 48 hours post KA. Complications surrounding the surgical implantation of the transmitters in this study seen at the beginning and throughout the experiments, resulted in reduced
number of animals being available for study, making the sample size number smaller than desirable. The ability to keep animals for up to 14 days following KA would allow for a better assessment of the potential cardiac damage that occurs as a result of seizure activity. This would allow for ECG monitoring to reveal any abnormalities that remain following seizure, as well as demonstrating the ability of the heart to either recover or succumb to further damage in the days following seizure induction.

**Conclusion**

Systemic administration of KA has been demonstrated to be a valid model of producing significant levels of seizure behavior in the rat. There is a clear relationship between seizure activity and cardiovascular disruption that is depicted by tachycardia, skipped beats and numerous HR parameter changes that occur during ictal periods. Based on the protection afforded by clonidine treatment, which was absent in atenolol treated animals, this study suggests that the cardiovascular pathology seen following KA administration is a result of CNS induced ANS modulation and not peripheral cardiac receptor binding. This study was in fact limited when attempting to find the exact link between such large systems of interest, the CNS and the heart. Refining the current techniques will undoubtedly provide the means by which to determine the exact relationship, as well as define the preventative measures required to lower the incidence of SUDEP. Despite these limitations, this study does however provide the first therapeutic intervention to attenuate cardiac damage consequent to seizure induction.
REFERENCES


