Effects of Anti-seizure Drugs on Motor Maps and Neurovascular Function

by

Haruna I. Dika

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

GRADUATE PROGRAM IN MEDICAL SCIENCE

CALGARY, ALBERTA

MAY, 2015

© Haruna I. Dika 2015
ABSTRACT

Seizures and epilepsy are known to alter motor map expression. Seizures also induce severe local brain hypoxia. Antiepileptics are drugs used to control seizures in patients with epilepsy. Most of these drugs suppress seizures without preventing or reversing epileptogenesis. Therefore, they are preferably called anti-seizure drugs (ASDs). ASDs inhibit neuronal activation and/or suppress synaptic or neuromuscular transmission to control seizures. Although these drugs alter neuronal and neuromuscular function, their effects on motor maps expression have not been explored. The role of ASDs on prevention of severe seizure-induced hypoxia (SISHE) is also unknown. Moreover, a loop diuretic, bumetanide, has shown anticonvulsant activity in neonates and is currently under clinical trials for managing neonatal seizures. Anti-seizure activity of bumetanide are thought to be through reversing GABA depolarizing effect in immature neurons, by inhibiting Na⁺,K⁺ and Cl⁻ co-transporter (NKCC1) which is the cellular chloride importer. Furthermore, bumetanide has recently reported to reduce seizure frequency in adult patients. Despite of these facts, study of bumetanide’s effects on motor maps expression is sparse and its role in controlling seizures in adults has not been given attention. In vivo experiments using young adult rats were carried out to determine the effects of several ASDs and bumetanide on motor maps expression. Intracortical microstimulation was done to derive the motor maps both pre- and post-drug application. Young adult rats were kindled and used to investigate the effects of bumetanide on seizure activity, which were measured 30 minutes post-bumetanide intraperitoneal treatment. Furthermore, kindling of rats was done to determine the effects of the ASDs and bumetanide on SISHE. Results showed that ASDs reduces the responsiveness of the motor cortex evidenced by forelimb map hypotrophy and elevated movement thresholds. This study has also demonstrated that bumetanide inhibits seizure activity
in matured rats, which implies that anticonvulsant activity of this drug is not exclusively due to blockage of NKCC1. Further findings show that with the exception of ethosuximide which reduced the expression of SISHE, other ASDs and bumetanide neither affected brain oxygen level nor severity of SISHE.
Body parts movement responses due to stimulation of different areas of the motor cortex can be summarized as so-called motor maps. Scientists are interested in the motor maps to better understand the organization of motor cortex and as a measure of plasticity within this brain region. Human and animal studies have shown that seizures alter motor maps leading to lower movement thresholds and larger motor maps (for review see Teskey et al., 2008) and more recently it has been discovered that seizures induce severe local brain hypoxia, termed seizure induced severe hypoxic episode (SISHE), an effect that is unrelated to the reduction in cardiac output and systemic blood pressure. There are many drugs used to treat seizures by affecting the neuronal function but their effects on motor map expression have not been explored. Likewise, the effects of these drugs on prevention or reduction of SISHE are unknown. Moreover, a diuretic drug bumetanide, has been found to have anticonvulsant activities, and currently it is under clinical trials for managing neonatal seizures. Like the classic anti-seizure drugs, bumetanide’s effects on motor maps expression and SISHE are unknown. Therefore, the theme of this thesis is to describe the effects of classic anti-seizure drugs and bumetanide on motor maps expression and their role in prevention of SISHE.

The thesis is presented in five chapters. Chapter one defines and classifies seizures and epilepsies. It also describes in brief the mechanisms of action of some anti-seizure drugs and outlines which parts of the cerebral cortex are considered the motor cortex as well as giving a brief description of how the motor maps were discovered. Additionally, it gives a review of some studies which have shown the effect of seizures and epilepsies on the expression of the motor maps. Chapter two describes a series of novel experiments aimed at determining the effects of
anti-seizure drugs on the motor maps expression while chapter three describes the experiments which aimed to show effects of the putative anti-seizure drug, bumetanide on the same in addition to description of the effects of this drug on the neocortical layer V pyramidal cells activity. Chapter four of this thesis describes experiments which were performed to demonstrate the effects of anti-seizures and bumetanide on SISHE. Lastly, chapter five gives a summary of the research results and draws general conclusion.
ACKNOWLEDGEMENTS

Special thanks go to my supervisor, Dr. G. Campbell Teskey for funding (through his supervisory grant) my training as well as for his invaluable guidance and support that has led to the completion of this work. Thanks to my supervisory committee members; Dr. Kenneth Lukowiak, Dr. Benedikt Hallgrimsson and Dr. Paolo Federico for their dedication, guidance and supervision and encouragement. A lot of thanks go to Bonita Gunning and Jordan Ferrell for their great assistance and technical support. Thanks to Ahmed Hussin, Dr. Jeffrey A. Boychuk and Omid Javizian for their data which I have included in developing Chapter three.

I am grateful to my beloved wife, Saada Mumba, for taking care of our kids, her support, patience and understanding while I was away studying. Thanks to the family of Dr. G. Campbell Teskey for their love and support they showed to me during my stay in Calgary. My appreciation also goes to Kathleen Scullion, Dr. Andrew Brown, Justin Rodych, Anna Singleton, Jeffrey Grab, Laura Ansell, Tania Bhullar, Marshal Wolff, Amanda Aitken, Jordan Robinson, Rachel Wang, Dr. Simon Spanswick, Ryan McCarthy, Tom Seredynski, Alex Kim, Luc Boutin and Gerry Coughlin for the cooperation and assistance rendered to me when working in Dr. Teskey’s laboratory. More gratitude goes to Dr. Jennifer Hatfield, the Associate Dean of the University of Calgary Cumming School of Medicine Global Health and International Partnerships, for her office funding my Tanzania/Calgary travel costs. Likewise, I am thankful to the University of Calgary International for partly funding my tuition and Tanzania/Calgary travel costs. Last but not least, I would like to thank God for giving me the strength and courage throughout the period of my studies.
To

Dr. G. Campbell Teskey

I could not have done this without your support

My family

For your patience and understanding while I was away for studies and for your love

This work is a sign of my love to you!

My parents, Amina Muna and Ismail Dika

In memoriam!
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................ ii
PREFACE........................................................................................................................................ iv
ACKNOWLEDGEMENTS ................................................................................................................... vi
DEDICATIONS ................................................................................................................................... vii
TABLE OF CONTENTS ..................................................................................................................... viii
LIST OF FIGURES ............................................................................................................................ xiii
LIST OF SYMBOLS AND ABBREVIATIONS ...................................................................................... xv

## CHAPTER ONE: GENERAL INTRODUCTION .............................................................. 1

1.1 Introduction ................................................................................................................................. 1

1.2 Seizures and Epilepsy .................................................................................................................... 3

1.2.1 Seizures and Epilepsy Defined .................................................................................................. 3

1.2.2 Classification of Seizures ......................................................................................................... 6

1.3 Anti-seizure Drugs ....................................................................................................................... 11

1.4 Motor Cortex ............................................................................................................................... 17

1.4.1 Cerebral Cortex ....................................................................................................................... 17

1.4.2 What is the Motor Cortex? ..................................................................................................... 20

1.4.3 Discovery of the Motor Cortex ............................................................................................... 21

1.5 Seizures, Epilepsy and Motor Maps ............................................................................................ 32

1.5.1 Motor Maps ............................................................................................................................ 32

1.5.2 Effects of Epilepsy on Motor Maps ....................................................................................... 34
1. 5.3 Laboratory Animal Studies on Motor Maps ................................................................. 36

1.6 Seizure Induced Severe Hypoxic Episodes (SISHE) ......................................................... 44

1.7 Thesis Objectives and Hypothesis ..................................................................................... 50

CHAPTER TWO: EFFECTS OF CLASSIC ANTI-SEIZURE DRUGS ON MOTOR
MAPS EXPRESSION .................................................................................................................. 52

2.1 Introduction .......................................................................................................................... 52

2.2 Methodology ....................................................................................................................... 54

2.2.1 Subjects .......................................................................................................................... 54

2.2.2 Procedures ...................................................................................................................... 54

2.2.3 Data analysis .................................................................................................................. 57

2.3 Results ................................................................................................................................ 58

2.3.1 Effect of Phenytoin on Motor Maps Expression ............................................................... 58

2.3.2 Effects of Ethosuximide, Valproate and Levetiracetam on Motor Maps Expression .. 62

2.3.3 Effect of Topiramate on Motor Map Expression .............................................................. 70

2.4 Discussion ........................................................................................................................... 74

CHAPTER THREE: ACUTE EFFECTS OF BUMETANIDE ON KINDLED SEIZURES,
MOTOR MAP EXPRESSION AND LAYER V PYRAMIDAL CELLS ACTIVITIES OF
YOUNG ADULT RATS .............................................................................................................. 80

Abstract .................................................................................................................................... 81

3.1 Introduction .......................................................................................................................... 83

3.2 Materials and Methods ....................................................................................................... 86

3.2.1 Animals .......................................................................................................................... 86
3.2.2 Implantation of Electrodes for Kindling......................................................... 86
3.2.3 Standard Kindling......................................................................................... 87
3.2.4 Testing Bumetanide Effects on ADTs, ADDs and Seizure Severity .............. 88
3.2.5 3Hz- Kindling .............................................................................................. 88
3.2.6 Craniotomy and Intracortical Microstimulation............................................ 89
3.2.7 Testing Bumetanide Effects on MTs and Forelimb Map Areas .................. 89
3.2.8 Slice Preparation ......................................................................................... 89
3.2.9 Electrophysiological Recordings .................................................................. 90
3.2.10 Testing Bumetanide Effects on Layer V pyramidal Cells ......................... 91
3.2.11 Statistical Analysis ................................................................................... 92
3.3 Results ............................................................................................................ 92
  3.3.1 Effects of Bumetanide on ADTs, ADDs and Seizure Severity ...................... 92
  3.3.2 Effects of Bumetanide on Motor Maps Expression in the neocortex ............ 93
    3.3.2.1 Effects of Bumetanide on Forelimb Representations ............................... 93
    3.3.2.2 Effects of Bumetanide on MTs ............................................................... 96
  3.3.3 Effects of Bumetanide on Layer V pyramidal cells .................................. 101
3.4 Discussion ...................................................................................................... 105
3.5 Acknowledgements ....................................................................................... 110

CHAPTER FOUR: EFFECTS OF ANTI-SEIZURE DRUGS ON SEIZURE INDUCED
SEVERE HYPOXIC EPISODE (SISHE).................................................................. 111

  4.1 Introduction .................................................................................................... 111
4.2 Methods ........................................................................................................................................... 113

4.2.1 Subjects ...................................................................................................................................... 113

4.2.2 Implantation of Electrodes and Oxygen Probes ........................................................................ 113

4.2.3 Seizure Inductions and Oxygen Recordings .............................................................................. 114

4.2.4 Testing the effects of ASDs on SISHE ....................................................................................... 115

4.2.5 Statistical Analysis ................................................................................................................. 117

4.3 Results .......................................................................................................................................... 117

4.3.1 Effects of Nifedipine on SISHE and Seizure Duration ................................................................. 117

4.3.2 Effects of Phenytoin on SISHE and Seizure Duration ................................................................. 120

4.3.3 Effects of Phenobarbital on SISHE and Seizure Durations ...................................................... 120

4.3.4 Effects of Ethosuximide on SISHE and Seizure Duration ......................................................... 120

4.3.5 Effects of Valproate on SISHE and Seizure Duration ............................................................... 127

4.3.6 Effects of Levetiracetam on SISHE and Seizure Duration ....................................................... 127

4.3.7 Effects of Topiramate on SISHE and Seizure Duration ............................................................ 132

4.3.8 Effects of Lamotrigine on SISHE and Seizure Durations ......................................................... 132

4.3.9 Effects of Bumetanide on SISHE and Seizure Duration ............................................................ 132

4.3.10 Summary of the Study Findings ............................................................................................. 139

4.4 Discussion ...................................................................................................................................... 142

CHAPTER FIVE: SUMMARY OF THE FINDINGS AND GENERAL CONCLUSION 146

5.1 Introduction ................................................................................................................................... 146

5.2 Summary of the Study Findings and General Discussion ............................................................. 147
LIST OF FIGURES

Figure 1-1: Seizure afterdischarge ................................................................. 5
Figure 1-2: Cerebral hemisphere lobes .......................................................... 18
Figure 1-3: Motor maps of of higher primates ............................................... 25
Figure 1-4: Seizure induced severe hypoxic episode ...................................... 47
Figure 1-5: Seizure induced severe hypoxia and reduced local cerebral blood flow...... 49
Figure 2-1: Relatively unchanged forelimb map sizes in response to phenytoin......... 59
Figure 2-2: Increased movement thresholds caused by phenytoin .................... 61
Figure 2-3: Representative forelimb maps post-saline, ethosuximide, valproate and LEV...... 63
Figure 2-4: Reduction of forelimb areas in response to ethosuximide, valproate and levetiracetam .................................................................................................................. 65
Figure 2-5: Increased movement thresholds caused by ethosuximide, valproate and levetiracetam .................................................................................................................. 68
Figure 2.6: Effects of topiramate on forelimb area in the neocortex ...................... 71
Figure 2.7: Increased movement thresholds caused by topiramate.......................... 73
Figure 3-1: Increased ADT and reduced seizure severity by bumetanide with no significant change in ADD.................................................................................................................. 94
Figure 3-2: Lack of gross changes in forelimb map in response to bumetanide treatment...... 97
Figure 3-3: Increased movement thresholds by bumetanide.................................... 99
Figure 3-4: Inhibition of cortical layer V pyramidal cells by bumetanide............... 102
Figure 3-5: Reduced number of APs fired to an intracellular current injection post-bumetanide treatment.............................................................................................................. 104
Figure 4-1: Prevention of SISHE by nifedipine ................................................................. 118
Figure 4-2: Seizure induced hypoxia and seizure durations post-phenytoin treatment ....... 121
Figure 4-3: Unchanged SISHE severity in response to phenobarbital................................. 123
Figure 4-4: Reduction in SISHE severity caused by ethosuximide ................................. 125
Figure 4-5: Effect of valproate on SISHE severity and seizure durations ......................... 128
Figure 4-6: Unchanged SISHE severity in response to levetiracetam ............................... 130
Figure 4-7: Non-significant change in SISHE severity and seizure duration post-topiramate 133
Figure 4-8: Seizure induced hypoxia and seizure durations post-lamotrigine ..................... 135
Figure 4-9: Lack of changes in SISHE severity and seizure durations post-bumetanide ....... 137
Figure 4-10: Effects of ASDs on seizure induced hypoxia and seizure durations .............. 140
# LIST OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation/Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AD</td>
<td>Afterdischarge</td>
</tr>
<tr>
<td>ADD</td>
<td>Afterdischarge duration</td>
</tr>
<tr>
<td>ADT</td>
<td>Afterdischarge threshold</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>ASD</td>
<td>Anti-seizure drug</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>CBZ</td>
<td>Carbamazepine</td>
</tr>
<tr>
<td>CFA</td>
<td>Caudal forelimb area</td>
</tr>
<tr>
<td>Cl</td>
<td>Chloride ion</td>
</tr>
<tr>
<td>CMN</td>
<td>Corticomotorneuron</td>
</tr>
<tr>
<td>CoCaTotIIa</td>
<td>Commission on Classification and Terminology of the International League Against Epilepsy</td>
</tr>
<tr>
<td>CSP</td>
<td>Cortical stimulation period</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>CST</td>
<td>Corticospinal tracts</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalography</td>
</tr>
<tr>
<td>EMG</td>
<td>Electromyography</td>
</tr>
<tr>
<td>EPP</td>
<td>End-plate potential</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance image</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-Aminobutyric acid</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>ICE</td>
<td>International Classification of Epilepsies and Epileptic Syndrome</td>
</tr>
<tr>
<td>ICES</td>
<td>International Classification of Epileptic Seizures</td>
</tr>
<tr>
<td>ICMS</td>
<td>Intracortical microstimulation</td>
</tr>
<tr>
<td>ILAE</td>
<td>International League Against Epilepsy</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>KCC2</td>
<td>Neuron-specific K⁺-Cl⁻ cotransporter (neuron K⁺ and Cl⁻ exporter)</td>
</tr>
<tr>
<td>LEV</td>
<td>Levetiracetam</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LTG</td>
<td>Lamotrigine</td>
</tr>
<tr>
<td>MEPP</td>
<td>Miniature end-plate potential</td>
</tr>
<tr>
<td>MR</td>
<td>Metabolic rate</td>
</tr>
<tr>
<td>MT</td>
<td>Movement threshold</td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Sodium ion</td>
</tr>
<tr>
<td>NKCC1</td>
<td>Na&lt;sup&gt;+&lt;/sup&gt;-K&lt;sup&gt;+&lt;/sup&gt;-2Cl&lt;sup&gt;-&lt;/sup&gt; cotransporter (cellular Na&lt;sup&gt;+&lt;/sup&gt;, K&lt;sup&gt;+&lt;/sup&gt;, and Cl&lt;sup&gt;-&lt;/sup&gt; importer)</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid or N-Methyl-D-aspartate</td>
</tr>
<tr>
<td>PB</td>
<td>Phenobarbital</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PG:E:W</td>
<td>Propylene glycol, ethanol and water in ratio of 4:1:5</td>
</tr>
<tr>
<td>PHT</td>
<td>Phenytoin</td>
</tr>
<tr>
<td>pO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Oxygen partial pressure</td>
</tr>
<tr>
<td>rCBF</td>
<td>Regional cerebral blood flow</td>
</tr>
<tr>
<td>RFA</td>
<td>Rostral forelimb area</td>
</tr>
<tr>
<td>RMP</td>
<td>Resting membrane potential</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>SISHE</td>
<td>Seizure induced severe hypoxic episodes</td>
</tr>
<tr>
<td>SMA</td>
<td>Supplementary motor area</td>
</tr>
<tr>
<td>TMS</td>
<td>Transcranial magnetic stimulation</td>
</tr>
<tr>
<td>$\mu$A</td>
<td>micro ampere</td>
</tr>
<tr>
<td>$\mu$m</td>
<td>micro meter</td>
</tr>
<tr>
<td>$\mu$s-</td>
<td>micro second</td>
</tr>
<tr>
<td>$\Omega$</td>
<td>Ohm</td>
</tr>
</tbody>
</table>
CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Seizures cause the reorganization of brain structure (Holmes, 2002; Holmes et al., 2002) and function. For instance human and animal studies have shown that seizures and epilepsies alter movement representations in the motor cortex (motor maps), leading to lower movement thresholds and larger motor maps (Teskey et al., 2008) that is related to altered movement (Henry et al., 2008). They also alter brain metabolism such as upregulation of cyclooxygenase-2 (Friedman and Dingledine, 2011), leading to various neurological symptoms. More recently, it has been discovered that seizures induce severe local brain hypoxia, an effect that is unrelated to the reduction in cardiac output and systemic blood pressure (Teskey and Farrell, 2012), the effect that may be one of the major causes of neurological deficits among epileptic patients. This thesis describes the role of anti-seizure medications on the expression of motor maps and the post-seizure hypoxic episode.

Antiepileptic drugs are the first line drugs in managing seizures associated with epilepsy. These drugs provide only symptomatic relief as they suppress seizures by raising seizure thresholds and have no ability to prevent or reverse epileptogenesis (Schachter, 2002; Shinnar and Berg, 1996; Temkin, 2001). Therefore, antiepileptic drugs are better called anti-seizure drugs (ASDs). Clinicians usually abbreviate antiepileptics as AEDs, but ASDs are also used in animal research thus referring to anti-seizure drugs. ASDs will be used throughout in this thesis.

The mechanisms of action of most ASDs, particularly the newer ones, are poorly understood. Currently research efforts are focused on identifying their mechanisms of actions on
ion channels, receptors and neurotransmitters synthesis and release, as well as on the efficacy and safety profiles of these drugs. Although the mechanisms of actions of some of the ASDs are not fully known, these drugs control seizures by altering neuronal function. However, how ASDs effect motor map expression has not been explored and their effects on seizure-induced hypoxic episodes are completely unknown. Interestingly, the most potent loop diuretic (bumetanide) which has been in clinical practice for many decades for managing fluid overload has in recent years found to have anti-seizure effects in neonates (Kahle et al., 2009; Mareš, 2009; Mazarati et al., 2009). Although it is not clear how bumetanide works to control seizures, its mechanisms of action are thought to be a result of blockage of the chloride transporter, the Na⁺-K⁺-2Cl⁻ co-transporter (NKCC1), which is the isoform of the NKCC2 co-transporter that is highly expressed in the thick ascending limb of the loop of Henle where the drug induces its diuretic effects.

NKCC1 is highly expressed in immature neurons but its expression decreases with age (Dzhala et al., 2005; Payne et al., 2003; Wang et al., 2013). NKCC1 accumulates chloride ions in immature neurons making them depolarize in response to activation of GABA_\text{A} receptors due to efflux of negatively charged chloride ions (Payne et al., 2003), which is different from adult neurons where GABA has inhibitory effect (Kaila, 1994). Blockage of NKCC1 by bumetanide in immature neurons reverses the GABA depolarizing effect by preventing intracellular Cl⁻ ion accumulation. In more recent development, in one study bumetanide was found to reduce seizure frequency in adult patients (Eftekhari et al., 2013). Unfortunately the role of bumetanide in control of seizures in adults has not been given attention. There is also limited information about the effects of bumetanide on motor maps expression.

Therefore, this thesis examined the effects of the ASDs on the neuronal networks (motor maps) and neurovascular functions. The thesis also examined whether bumetanide has
anti-seizure activity in adult or not. *In vivo* experiments using young adult rats were carried out to determine the effects of ASDs and bumetanide on the movement thresholds and forelimb motor maps. Furthermore, the effects of the ASDs and bumetanide on seizure-induced hypoxic episodes were tested. The thesis aimed to answer the question of whether acute ASD and bumetanide administrations reduce the responsiveness of the motor cortex (or not) and to demonstrate the effects of these drugs on seizure induced severe hypoxic episodes (SISHE).

I begin by giving a brief description of seizures and epilepsies, anti-seizure medication, motor cortex and effect of seizures and epilepsies on the expression of the motor maps as well as describing seizure induced severe hypoxic episode.

### 1.2 Seizures and Epilepsy

#### 1.2.1 Seizures and Epilepsy Defined

The term seizures may be used in various contexts to describe sudden and severe physical or psychological event. In this thesis, this word will be used referring to *epileptic seizures* which are abnormal and usually excessive or hypersynchronous neuronal activity in the brain (Fisher et al., 2005). The abnormal neuronal electrical discharge can be recorded by electroencephalography (EEG). The precise mechanisms underlying seizure initiation and propagation are not yet fully known. While seizures likely originate in one area of the brain, they can often spread to other brain regions. As a result, seizure location contributes to the nature of the behavioral symptoms. These behavioral disruptions can include changes in motor function either during or between seizures. While epileptic seizures can be diagnosed by clinical symptoms and signs and/or by EEG, it has proved difficult to develop one unifying classification scheme for them. This difficulty arises from the wide diversity of both seizure symptoms as well
as underlying causes of seizures. Seizures can be provoked by a number of factors including, metabolic disturbances like hypoglycemia, electrolyte imbalance and hypoxia, high fever, drug intoxication, head injury, infections like encephalitis and meningitis, intracranial bleeding and space occupying lesions in the brain.

Epilepsy refers to the chronic neurological condition that is characterized by increased predisposition for recurrence of epileptic seizures, and is often accompanied with neurobiological, cognitive and psychosocial impairment (Fisher et al., 2005). Occurrence of at least one epileptic seizure is required for defining epilepsy. In epilepsy seizures may be provoked or may occur without any apparent provocation. Epilepsy is associated with the reduction of threshold for elicitation of seizure known as afterdischarge threshold (ADT) and prolonged afterdischarge duration (ADD). Afterdischarge is the rhythmic impulse generation by neurons after termination of stimulation. Figure 1-1 below demonstrates an afterdischarge. Epilepsy is a common disease affecting at least 50 million people worldwide and has a high socioeconomic burden (Begley et al., 2000).
Figure 1-1: Seizure afterdischarge

Figure 1-1 is an EEG trace illustrating a hippocampal primary afterdischarge (which is labeled as $I^0$ afterdischarge) in a kindled rat.
1.2.2 Classification of Seizures

The sites where seizures originate and the extent of their propagation to other brain regions determine the symptoms and signs that are produced. This can range from simple brief muscle twitches or short-lived loss of attention to full blown convulsions with or without loss of consciousness. Several attempts have been made to establish a universal taxonomy to classify epileptic seizures. Since 1970s, a number of classification schemes for epileptic seizures have been suggested by Commission on Classification and Terminology of the International League Against Epilepsy (CoCaTotIla) as well as by individual scholars (Berg et al., 2010; CoCaTotIla, 1981; CoCaTotIla, 1989; Dreifuss et al., 1985; Engel, 2001; Engel, 2006; Gastaut, 1970; Luders et al., 1998; Merlis, 1970; Wolf, 2003). Three of the proposed classification systems have gained the most attention. These are; the International Classification of Epileptic Seizures (CoCaTotIla, 1981), the International Classification of Epilepsies and Epileptic Syndrome (CoCaTotIla, 1989) and the 2010 Classification Scheme (Berg et al., 2010).

The International Classification of Epileptic Seizures (ICES) was first adopted in 1981. This system categorized seizures depending on clinical signs and symptoms, the location of the epileptic discharges in the cortex and the extent and pattern of the propagation of the epileptic discharge in the brain as evidenced by the EEG (CoCaTotIla, 1981). Under this system epileptic seizures were classified into two major groups; partial and generalized. Partial seizures are localized to focal area of one cerebral hemisphere while generalized seizures are more distributed to involve both hemispheres and present with bilateral motor manifestations (CoCaTotIla, 1981). Partial seizures were further classified by the extent that consciousness is disrupted. Simple partial seizures do not involve a loss of consciousness while complex partial
seizures do involve some loss of consciousness. Another category of partial seizures is “partial seizures evolving to secondarily generalized seizures”, in which either simple partial or complex partial seizures progress to generalized tonic or clonic or tonic-clonic convulsions (CoCaTotlla, 1981). Generalized seizures, which may be convulsive or non-convulsively were further subdivided into tonic, myoclonic, clonic, atonic, tonic-clonic and absence seizures (CoCaTotlla, 1981). Tonic seizures are generalized seizures that are characterized by sustained increase in muscle contraction lasting for few seconds to minutes. Tonic seizures usually fix the limbs in tense position and are associated with deviation of the eyes, head or whole body toward one side. Myoclonic seizures (myoclonic jerks) involve both cerebral hemispheres and consist of rapid, brief (lasting less than a second) involuntary contractions of axial, proximal or distal limb muscle or muscle groups. Clonic seizures have prolonged myoclonuses which are regularly repetitive and involve the same group of muscles. Atonic seizures are generalized seizures that are characterized by sudden loss of (trunk, limb, head or jaw) muscle tone which is not preceeded by tonic or myoclonic event (Blume et al., 2001; CoCaTotlla, 1981). Generalized tonic-clonic seizures are characterized by bilateral tonic extension of the extremities lasting for few seconds followed by bilateral clonic contraction and usually associated with autonomic disturbances (Blume et al., 2001). Absence seizures are a type of generalized seizures, characterized with brief (less than 20 seconds) episodes of impaired consciousness with EEG characteristics of generalized spike-and-slow wave discharges (Panayiotopoulos, 2008; CoCaTotlla, 1981). According to the ICES 1981, seizures which do not fit into any of the groups were labeled as unclassified.

Many patients experience seizures that change with duration of the illness and sometimes one patient may have more than one type of seizure at a time or in sequence. Because of the
inability to properly classify all epileptic seizures, in 1989 the International League against Epilepsy (ILAE) adopted the International Classification of Epilepsies and Epileptic Syndrome (ICE). The epileptic syndrome was defined as an epileptic disorder characterized by a constellation of symptoms and signs occurring together without necessarily having similar aetiology or prognosis (Engel, 2001).

The 1989 ICE classification has been used concurrently with the 1981 ICES classification. However, the 1989 ICE did not subdivide the focal seizures into either simple or complex (CoCaTotIlia, 1989; Engel, 2006). The 1989 ICE classified epilepsies and epileptic syndromes into four groups: These are localization related epilepsies and syndromes, generalized epilepsies and syndromes, epilepsies and syndrome which are undetermined whether localized or generalized and Special Syndromes (CoCaTotIlia, 1989). The localization-related epilepsies and syndromes incorporated focal seizures as described by the 1981 ICES. According to the 1989 ICE, generalized epilepsies and syndromes are epileptic disorders with generalized seizures with an evidence of both hemispheres involvement. The localization related and generalized epilepsies and syndromes were each subdivided into symptomatic or secondary (whose causes are known), cryptogenic (with hidden or occult cause) and idiopathic with no underlying cause (CoCaTotIlia, 1989). The idiopathic cases likely have a genetic component and they are defined by age related onset as well as clinical and characteristic EEG pattern. Epilepsies and syndromes undetermined whether focal or generalized are those whose placement into either focal or generalized cannot be done. For example when a patient gets both focal and generalized seizures at the same or different times or when seizures occur during sleep in which the positive signs of either focal or generalized seizure onset can’t be made. Special syndromes include situation-
related seizures such as febrile convulsions and seizures that occur only when there is an acute metabolic disturbance.

The 1989 ICE classification system was not only complex but also failed to classify some patients into a specific syndrome. As a result, the 1989 ICE classification system has been viewed as incomplete and several improvements were proposed (Berg et al., 2010; Engel, 2001; Luders et al., 1998; Engel, 2006). With increasing knowledge of seizures a new classification scheme proposed in 2010 is in place. The 2010 classification is simple and more flexible than the previous classifications. According to the 2010 classification scheme, seizures can be classified using: mode of seizure onset, etiology, syndromic approach or other dimensions (Berg et al., 2010; Berg et al., 2009). The 2010 scheme maintained the use of focal and generalized seizures as described by the 1981 ICES to indicate the site seizures originate. However it abandoned the categorization of focal seizures as simple or complex. Despite of abandoning the use of terms simple partial and complex partial which were used in 1981 ICES because they were seem imprecise, the 2010 classification scheme continues to subdivide focal seizures on the basis of level of consciousness during seizures. This subdivision is viewed as non-scientific, but it is important for practical and social purposes (Berg et al., 2009; Berg et al., 2010). For instance restriction of driving can be based on association of loss of consciousness. Focal seizures without loss of consciousness or responsiveness (which roughly corresponds to simple partial seizures as used in 1981 ICES) are classified as “focal motor” or “focal autonomic” depending on the seizure manifestations and focal involving subjective sensory or psychic phenomena only (aura). Focal seizures with impairment of consciousness or responsiveness (which roughly matches with the concept of complex partial seizure) are classified as ‘Dyscognitive focal seizures’ (Berg et al., 2009; Berg et al., 2010; Blume et al., 2001). Another category of focal
seizures is “focal evolving to a bilateral, convulsive”. This replaces “secondarily generalized seizures”, the term which was used inconsistently in 1981 ICES and it was not clearly understood by most people.

Spasms are neither classified as focal nor generalized, but treated as a separate group. Additionally, the neonatal seizures are not classified separately from adult seizures and categorization of generalized seizures has been simplified compared to the 1981 ICES. The 2010 classification scheme categorizes generalized seizures into six groups, namely; tonic seizures, clonic seizures, tonic-clonic seizures (in any combination), atonic seizures, absence seizures and myoclonic seizures. Absence seizures are further subdivided into typical, atypical and absence seizures with special features which are myoclonic absence and eyelid myoclonia. Likewise, myoclonic seizures are subdivided into myoclonic, myoclonic atonic and myoclonic tonic (Berg et al., 2009; Berg et al., 2010).

Regarding etiological classification, the 2010 scheme substitutes the use of symptomatic, idiopathic and cryptogenic epilepsies used in the 1989 ICE with structural and metabolic, genetic epilepsies and epilepsies of unknown causes respectively. According to the syndromic approach, epilepsies and syndromes can be put in four groups; namely electro-clinical syndromes, epilepsies secondary to specific cause, constellations and epilepsies of unknown causes. The scheme discourages the use of syndrome and epilepsy interchangeably as used in 1989 ICE. Syndrome (electro-clinical syndrome) is redefined to refer to a group of symptoms and signs that are consistently identified by a bunch of electro-clinical characteristics. Other clusters of seizure disorders that are not recognized as electro-clinical syndromes are grouped as constellations.
Other dimensions like age at onset of epilepsy and natural evolution can also be used to classify epilepsies (Berg et al., 2010; Berg et al., 2009).

1.3 Anti-seizure Drugs

Anti-seizures also called anticonvulsants and antiepileptic drugs are drugs which are used to control seizures in people with epilepsy. Anti-seizure drugs (ASDs) include phenytoin (PHT), phenobarbital (PB), carbamazepine (CBZ), ethosuximide, valproate, topiramate, lamotrigine (LTG) and levetiracetam (LEV) to mention a few. Epilepsy pharmacological treatment has a long history, starting from 1850s when bromide salts were introduced in managing seizures (Krall et al., 1978; Shorvon, 2009). In 1912, PB was found to have anticonvulsant activity and became the drug of choice for many years (Shorvon, 2009), and in relatively recent years PB derivatives such as primodone were developed. In 1940, PHT was found to be an effective drug for the treatment of epilepsy and since then it became a major first-line ASD in the treatment of focal seizures. Ethosuximide was introduced into clinical practice in 1960 and has been the drug of choice for children with absence seizures (Leppik, 2001). CBZ and Valproate were approved as ASDs in 1970s (Krall et al., 1978). These ASDs were the mainstays of seizure treatment until the 1990s, when newer ASDs such as topiramate, LTG and LEV with a number of potential advantages over older drugs were developed. The newer ASDs have relatively fewer side effects, better tolerability and fewer drug interactions and they lack need of blood level monitoring (Johannessen Landmark and Patsalos, 2010; McCabe, 2000; Perucca, 2009; Perucca et al., 2007). Additionally, bumetanide, which is a diuretic drug, has proven to have anticonvulsant activity and currently it is under clinical trials for managing neonatal seizures.
Using a variety of mechanisms, the ASDs suppress seizure activity. Even though the mechanisms used by some ASDs are unknown, the known mechanisms include inhibition or potentiation of receptors, ionic channels or ion transporters and alteration of neurotransmitters levels. The differences in the mechanisms of actions of these drugs probably explain the differences in the effectiveness of these drugs on different seizure types. Additionally, it is expected that different seizure types involve different mechanisms and therefore respond differently to ASDs.

PHT which has been used for more than 60 years; and CBZ are known to be effective in treating partial seizures and generalized seizures of primarily the tonic-clonic type (De Silva et al., 1996). Both PHT and CBZ act by stabilizing the inactivated state of voltage-gated sodium channels, making neurons less excitable (LaRoche and Helmers, 2004; Macdonald, 1989; McLean and Macdonald, 1986b; McLean and Macdonald, 1986a; Schwarz and Grigat, 1989; Wakamori et al., 1989; Yaari et al., 1986). The reduction in the activity of voltage-gated sodium channels appears to exhibit a property of use-dependence, in which the drugs preferentially block the excitation of the cells that are firing repetitively. Interestingly, these drugs are thought to reduce the rate of discharge of neurons without reducing the amplitude and duration of individual action potentials (McLean and Macdonald, 1986a). Both PHT and CBZ may also act by enhancing GABA-mediated inhibition (Granger et al., 1995; Macdonald and McLean, 1982). In addition to these mechanisms, PHT reduces both neuronal calcium uptake (Macdonald and McLean, 1982; Messing et al., 1985) and excitatory synaptic transmission in the hippocampus (Griffith and Taylor, 1988).
PB is among the oldest ASDs and is equally used for the treatment of generalized and partial seizures. Although PB effectively controls both generalized and partial seizures, its use is limited by its sedating effects. PB has GABAergic effects, where it prolongs the duration of GABA-mediated chloride channel openings by binding at GABA<sub>A</sub>/benzodiazepine receptor complex (Twyman et al., 1989). Prolonged opening of chloride channels permits an increasing inflow of chloride ions across the membrane, causing neuronal hyperpolarization. Different from benzodiazepines which also bind on GABA<sub>A</sub> receptors causing increasing receptor opening frequency, PB increases the mean channel open duration of GABA<sub>A</sub> receptor currents without altering opening frequency (MacDonald et al., 1989; Study and Barker, 1981; Twyman et al., 1989).

Ethosuximide, an ASD effective for treating absence seizures modifies low-threshold T-type calcium currents (also called transient neuronal calcium currents) in thalamic neurons (Coulter et al., 1989a; Macdonald and Kelly, 1995; Meldrum, 1996), whose activity is linked to absence seizures (Coulter et al., 1989b).

Another ASD which is used in treating absence seizures is valproate (Davis et al., 1994). Experiments in rats have shown that, unlike ethosuximide which acts on the thalamic neurons, valproate reduces the low-threshold T-type calcium currents in the primary afferent neurons. For example, Kelly and his co-workers (1990) found that valproate at clinically acceptable concentrations reduces the low-threshold T-calcium current in rat nodose ganglion neurons. Valproate acts through other multiple mechanisms, which can explain why it is widely used to treat both focal and other types of generalized seizures apart from absence seizure (Davis et al., 1994).
Like PHT and CBZ, valproate reduces high-frequency neuronal firing by blocking sodium-dependent action potentials; as evidenced by its effects on the culture neurons of mouse brain (Macdonald and Kelly, 1993; Macdonald and Kelly, 1995; McLean and Macdonald, 1986b). Valproate may also attenuate seizures through GABA-mediated mechanism but this topic is controversial. Although some researchers doubt the effect of valproate on potentiation of GABA effects (Baldino and Geller, 1981), others have demonstrated that it enhances GABA effects presynaptically. Other studies have shown that, valproate causes significant increase in the GABA content of the brain by increasing GABA synthesis (Johannessen, 2000; LöScher, 1999) and inhibition of nerve terminal GABA-transminase by increasing succinic semialdehyde (Johannessen, 2000). GABA transaminase is the enzyme which is responsible for inactivating GABA. However, Farrant and Webster (Farrant and Webster, 1989) suggested that the acute depressant effect of valproate is most likely due to a potentiation of postsynaptic GABA receptor action rather than increasing the brain GABA level. In addition to the above described mechanisms, valproate also attenuates neuronal excitation induced by NMDA-type glutamate receptors (LöScher, 1999).

Topiramate is a relatively newly developed ASD and has been used as adjunctive therapy for the treatment of focal epilepsies (Ben-Menachem, 1997; French et al., 2004b; French et al., 2004a; Reife and Pledger, 1997) and generalized tonic clonic seizures (Glauser et al., 2007; Walker and Sander, 1996). Similar to valproate, many mechanisms have been suggested for topiramate’s ability to reduce seizures. Topiramate blocks voltage-dependent sodium channels (DeLorenzo et al., 2000; McLean et al., 2000). Topiramate also potentiates GABAergic receptors (White, 1997) by binding to another site apart from the GABA$_A$/Benzodiazepine receptor
complex. Its anticonvulsant effects may also be mediated by antagonizing NMDA–glutamate receptors (Meldrum, 1996; Shank et al., 1994).

LTG, another newly developed ASD, is used as adjunctive treatment of partial seizures in adults and children, as well as for adjunctive therapy for primary generalized tonic-clonic seizures (French et al., 2004b; Biton et al., 2005; Trevathan et al., 2006; Motte et al., 1997). LTG is also used for the treatment of newly diagnosed absence seizures in children (French et al., 2004a). Although the mechanisms of action of LTG are not fully known, experiments have shown that it blocks the repetitive firing neurons by inactivating voltage-dependent sodium channels. However, unlike other ASDs such as CBZ and PHT which act on voltage-sensitive sodium channels, LTG preferentially reduces firing rate of neurons that use glutamate and aspartate as neurotransmitters (Leach et al., 1986). It inhibits the release of these excitatory amino acid neurotransmitters (Wang et al., 1996). LTG not only controls seizures, but has neuroprotective effect (Papazisis et al., 2008), the effect that is thought to be due to inhibition of release of glutamate and aspartate, thus making it an important candidate in managing hypoxic-ischemic brain damage (Crumrine et al., 1997; Smith and Meldrum, 1995; Wiard et al., 1995).

While the mechanisms of some drugs like CBZ are relatively well understood, mechanisms of another newly discovered drug, LEV are poorly understood. Nevertheless the drug is thought to alter synaptic transmission through alteration of vesicle fusion (Yang et al., 2007) and modulation of GABA (Doelken et al., 2010). Despite its poorly understood mechanisms of action LEV is an attractive ASD because its pharmacodynamic and pharmacokinetic properties are more favourable as compared to other ASDs. It is rapidly absorbed following oral administration with excellent bioavailability and quick achievement of
steady-state concentrations. Additionally, it has minimal plasma protein binding and does not inhibit or induce hepatic enzymes making it with very low risk of drug interactions as compared to other ASDs (French and Arrigo, 2005; Glass et al., 2005; Hirsch et al., 2007; Otoul et al., 2007). LEV is used as adjunctive therapy to treat focal seizures, myoclonic seizures, primary generalized tonic-clonic seizures, and idiopathic generalized epilepsy (Berkovic et al., 2007; Doelken et al., 2010; French et al., 2004b; Glauser et al., 2006; Tsai et al., 2006). LEV is also effective for managing partial seizures in infants and young children (Pin a-Garza et al., 2009).

A high loop ceiling diuretic, bumetanide has been shown to express anticonvulsant effects in neonates. Currently bumetanide is in clinical trials as a prospective ASD in neonates. Mechanisms of action of bumetanide are not completely understood, but are largely thought to be a result of blockage of the two cation-chloride co-transporter (NKCC1). NKCC1 is the cellular chloride importer which is highly expressed in immature neurons but not in matured neurons (Dzhala et al., 2005; Payne et al., 2003; Wang et al., 2013). Gama amino butyric acid (GABA) which is known to inhibit neuronal firing by increasing an inwardly directed Cl⁻ conductance mediated by GABAₐ receptors, has depolarizing effect on immature brain neurons as well as in mature afferent, dorsal root ganglia and sympathetic ganglion neurons through intracellular accumulation of Cl⁻ ions (Alvarez-Leeffmans et al., 1988; Misgeld et al., 1986). Opening of chloride ion channels by GABA leads to Cl⁻ efflux and thus depolarization. Bumetanide by blocking NKCC1 lowers intracellular Cl⁻ ions and thus reverses the GABA depolarizing effect on GABAₐ receptors leading to inhibition. Experiments in rats have shown that, bumetanide decreases incidence of the tonic phase of generalized tonic-clonic seizures (Mareš, 2009) and inhibits seizure induction (Mazarati et al., 2009).
As mentioned earlier, the clinical features of seizures depend on the site of their origin in the motor cortex. It is therefore important to give a brief review of the anatomy of the motor cortex. This is described in the next section.

1.4 Motor Cortex

1.4.1 Cerebral Cortex

The motor cortex is part of the cerebral cortex, which is the outer portion of the cerebrum. The cerebrum is divided into diencephalon (thalamus and hypothalamus) and the telencephalon that forms the right and left hemispheres, connected by the corpus callosum (Snell, 2006). In humans, the surface of each cerebral hemisphere is arranged into folds called gyri that are separated from each other by fissures called sulci (Snell, 2006). The major sulci namely the central sulcus (Rolandic fissure), lateral sulcus (Sylvian fissure) and parietal-occipital sulcus, divide each cerebral hemisphere into four lobes: the frontal, parietal, temporal and occipital lobes (figure 1-2).

The frontal lobe lies anterior to the central sulcus and above the lateral sulcus. It is involved in initiation and control of the body voluntary movements, planning and modulation of emotions and personality (Chayer and Freedman, 2001; Penfield and Rasmussen, 1950). The parietal lobe which is placed posterior to the central sulcus and above the lateral fissure is associated with processing tactile sensory information such as pressure, touch, and pain (Frith and Blakemore, 2005; Penfield and Rasmussen, 1950). It also takes in auditory and visual signals and associates them with memories leading to meaning (Goodale and Milner, 1992; Mishkin and Ungerleider, 1982).
Figure 1-2: Cerebral hemisphere lobes

Figure 1-2: A sketch diagram showing cerebral hemisphere lobes from the lateral view. The figure was drawn on the basis of knowledge of human brain anatomy.
The temporal lobe is located below the lateral fissure. The temporal lobe contains the hippocampus which is important in formation of memories and areas involved in interpretation of sounds and the language (Smith and Kosslyn, 2007). The occipital lobe lies behind the parietal-occipital fissure. It processes visual information and analyses forms, colour and movements (Guyton and Hall, 1996b; Lennie, 2000; Wurtz and Kandel, 2000). There is the fifth cerebral lobe, the cingulate gyri or limbic lobe which is visible only when sagittal section of the brain is made and is marked by the cingulate sulcus. It should be pointed that, the sulci do not signify physiological boundaries; they are only landmarks.

The cerebral neocortex is made up of gray matter which in human contains about 10 billion neurons arranged into six layers (Snell, 2006). The six cortical layers from the surface inward are: molecular layer (I), outer granular layer (II), outer pyramidal layer (III), inner granular layer (IV), inner pyramidal layer (V) and multi-form layer (VI). The pre-central gyrus lacks internal granular layer (layer IV), and is therefore called agranular cortex to differentiate it from post-central gyrus which is called granular cortex. The inner pyramidal cell layer sometimes called the ganglion cell layer in the pre-central cortex contains giant pyramidal neurons (Betz cells). The inner granular layer is the major input of the cortex and the inner pyramidal cell layer is the output layer (Snell, 2006). Thus, the inner pyramidal cell layer is the site where corticospinal tracts (CST) originate. About 30 percent of the CST fibres originate from the pre-central cortex (Siegel and Sapru, 2010; Guyton and Hall, 1996a) with the giant pyramidal cells axons accounting about 10 percent of the CST fibres from the pre-central cortex or 2-3% of all CST fibres (Lasek, 1941; Rivara et al., 2003; Guyton and Hall, 1996a). The post-central cortex neurons contribute a significant number of axons in the CST. In human brain the contribution is about 40 percent (Siegel and Sapru, 2010; Guyton and Hall, 1996a). The rest of
CST fibres originate from the premotor and supplementary motor area [SMA] (Siegel and Sapru, 2010; Guyton and Hall, 1996a).

1. 4.2 What is the Motor Cortex?

Functionally the motor cortex can be described as the part of the cerebral cortex that is involved in the control of voluntary movement (Guyton and Hall, 1996a; Sessle and Wiesendanger, 1982). Stimulation of the motor cortex elicits movement which may range from simple twich to complex movement such as manipulation of objects (Sessle and Wiesendanger, 1982). Anatomically the motor cortex can best be defined as all regions of the six layered cerebral cortex which have corticospinal projections (Teskey and Kolb, 2011; Sessle and Wiesendanger, 1982), as opposed to the traditional definition which refers it as the agranular part of the cerebral cortex. The absence of an appreciable internal granular layer in the precentral cortex is merely a result of a developmental structural modification peculiar to this region of the cerebral cortex (Marin-Padilla, 1970). It has nothing to do with the motor functioning of the cerebral cortex. Thus the motor cortex includes areas across the central sulcus. This includes the primary motor cortex (Foerster, 1936) or Brodmann area 4, premotor cortex (Fulton, 1935) or Brodmann area 6 and the cingulate cortex (Fulton, 1935), SMA (Gould et al., 1986; Luppino et al., 1991; Mitz and Wise, 1987; Penfield and Rasmussen, 1950; Woolsey et al., 1951) and somatosensory cortex (Beevor and Horsley, 1887; Grunbaum and Sherrington, 1901; Penfield and Rasmussen, 1950; Ferrier, 1873; Ferrier, 1874) which occupies Brodmann areas 1, 2 and 3, provided they contain neurons which send CST projections.
Brodmann’s area 4, also known as the primary motor cortex, occupies a narrow strip anterior to the central sulcus. Brodmann’s area 6, which is referred to as the premotor area lies immediately anterior to Area 4. The area 6 is wide and is further subdivided into dorsal and ventral regions. The SMA is located on the superior and medial aspect of cerebral hemisphere. Brodmann’s areas 1, 2 and 3 are localized posterior to the central sulcus. In some animals, the term sensorimotor cortex is sometimes used because of the overlapping between motor and sensory functions of the neocortex.

1. 4.3 Discovery of the Motor Cortex

The Canadian neurosurgeon Wilder Penfield is well known for describing the somatotopic representation of the human body part movements in the primary motor cortex; the motor homunculus. Although Penfield is viewed as the father of the human motor map, studies of the motor cortex were performed many years before Penfield’s work. Emmanuel Swedenborg was probably the first person in 1744 to propose the topographic motor map in the brain, though his writings did not explain how he arrived to his conclusion. He argued that the cortex was the highest sensory and motor structure of the brain and stated that, the body movements are controlled in the cerebral cortex, with the face and feet being controlled by the lowermost and uppermost parts of the cerebral cortex respectively (Gross, 2007; Gross, 2009).

Apart from lost ideas of Swedenborg, the first well documented and crucial experiments in the discovery of the motor cortex were those conducted by the two young physicians of the Berlin Physiological Institute; Gustav Fritsch and Edvard Hitzig in 1870. Fritsch and Hitzig were the first to demonstrate that, the cerebral cortex is an excitable tissue and that it controls body movements. They did experiments by stimulating dogs’ cortices using direct battery current. In
their experiments, they discovered that electrical stimulation of distinct areas of the anterior part of the cortex elicits movement on the contralateral side of the body, and the different types of movement they observed varied depending on what part of the cortex was stimulated (Gross, 2007). When they destroyed the foreleg center, dogs lost control of coordinated movement of this body part (Gross, 2007). Their findings suggested that different body parts are represented on the cerebral cortex and each cortical region has specific function.

Later, Dr. John Hughlings Jackson reported that specific areas of the cerebral cortex control movements of different parts of the body while searching for causes of epilepsy. After noting a consistent systematic spread of convulsions from one body part to the next, he was convinced that different cortical sites must be involved in the control of different muscle groups and that these muscle groups are topographically organized in the cortex (Foerster, 1936; Gross, 2007). It is believed that Jackson had already speculated this fact before Hitzig and Fritsch experiments, though Hitzig and Fritsch published their report earlier than him. While Jackson wrongly believed that some movements are primarily controlled by centers in the striatum, he made significant contribution to the understanding of the organization of the motor cortex (Foerster, 1936; Graziano, 2009; Hitzig, 1900).

David Ferrier stimulating cortical surfaces of cats, rabbits, guinea pigs and monkeys with alternate electric current replicated the results of Fritsch and Hitzig experiments and gave a more descriptive motor map. Different from Fritsch and Hitzig observations, Ferrier noticed that the cortical area posterior to the central sulcus, in addition to the precentral gyrus can elicit contralateral limb movement and discovered that the body representation in the precentral gyrus is organized upside down (Ferrier, 1873; Ferrier, 1874). Additionally, David Ferrier discovered
other two distinct cortical regions (FEF and PEF) which control eye and head movements (Ferrier, 1874). Ferrier (1873) also demonstrated that the region which controls mouth and tongue movements in cats and dogs lies in the posterior part of the inferior frontal cortex. This region corresponds to the Broca’s area of the human brain. Ferrier (1873) also argued that the mouth movement is bilaterally controlled.

Charles E. Beevor and Victor Horsley gave detailed description of the motor maps suggested by Fritsch and Hitzig, Jackson and Ferrier. Beevor and Horsley’s experiments were focused on the forelimb representation in monkeys’ motor cortices. Beevor and Horsley (1887) observed the upper limb muscles being progressively represented in the motor cortex with the proximal limb muscles represented in the dorsal cortical region and distal muscle groups in the ventral region; and they noted a lack of an absolute line of demarcation between the areas of localization with each movement having a centre of maximum representation, which gradually fades off. Beevor and Horsley (1887) located specific areas which produced purposeful or complex movements like hand-to-mouth, reaching and defensive movements. Beevor and Horsley (1887) maps showed that there were motor points anterior and posterior to the central fissure (in sensory cortical area).

Sir Charles Sherrington and his collaborators did a number of experiments on the brains of chimpanzees, gorillas and orangutans, in efforts of understanding anatomy and functioning of the motor cortex. Among his collaborators are Alfred Grunbaum (who later changed his name to Alfred Leyton) and Graham Brown. Grunbaum and Sherrington (1901, 1903 and 1917) reported that, motor effects are elicited most readily by simulating precentral gyrus than on more anterior parts of the frontal cortex. Like Ferrier, Grunbaum and Sherrington observed some motor
responses following stimulation of the postcentral gyrus, and the eye movements when the frontal region of brain and extreme apex of the occipital lobe were stimulated. However, they hesitated to include these areas as part of the motor cortex (Grunbaum and Sherrington, 1901; Grunbaum and Sherrington, 1903; Leyton and Sherrington, 1917). Since injury to the precentral gyrus often does not align with the extent or duration of movement deficits it has been suggested that the primary motor cortex is not the only brain area that controls voluntary movement (Grunbaum and Sherrington, 1903). Brown and Sherrington (1912) noted that stimulation of one cortical site modifies the movement evoked in another site. This led them to suggest that the cortical sites are interconnected by fibers (Brown and Sherrington, 1912).

Cytoarchitecture differences between the anterior and posterior parts of the agranular cortex along with converging lines of evidence from clinical observations and cortical ablation experiments performed in monkeys, led Fulton (1935) and later, Foerster (1936) to propose that the motor cortex be divided into a primary motor area, restricted to the agranular cortex with giant pyramidal neurons, and a premotor area on the more anterior region. Fultons’s definitions were based on observations made by Walshe on monkeys after ablation of different areas of the motor cortex, which indicated that the two areas have different motor functions (Fulton, 1935). Foester (1936) made this conclusion by observing behavior following electrical stimulations of epileptic patients’ cortical surface during operation. Foerster (1936) speculated, that specific functions of the primary motor cortex is the isolated innervations of single muscle group and that of premotor cortex is innervations of all muscle groups of the opposite side of the body.
Figure 1-3: Body parts representation along the precentral gyrus of higher primates. [From: Grunbaum and Sherrington, 1901]
The term *premotor cortex* was later criticized, as it was seen a lack of a clear functional separation of the two histological areas (Walshe, 1935; Woolsey et al., 1951). Walshe (1935) was the first to oppose the use of this term claiming that, the observations made by Fulton were a result of brain lesions. Wilder Penfield and colleagues; and Woolsey et al (1951) boldly rejected the use of term *premotor cortex* by arguing that the differences observed by Fulton were caused by differences in representations of fingers, elbow, shoulder and trunk on anterior and posterior regions of the motor cortex. Despite these criticisms, the term is still in use, and others have gone to the extent of subdividing it into ventral and dorsal premotor cortices.

Penfield and colleagues’ reports were based on experiments Penfield conducted in patients with epilepsy that he operated on. Penfield, electrically stimulated discrete points on the cortical surfaces and noted the type of movement produced. In addition to the movement evoked, he also noted the patients’ reports of induced sensation. Penfield and his students and colleagues, used stimulation maps from Penfield’s surgeries to develop a generalized complete map of the human motor cortex, known as the *homunculus* (Penfield and Rasmussen, 1968). These studies demonstrated that body parts are represented in an upside down manner on the contralateral precentral gyrus. The Penfield’s *motor homunculus* shows that, the body parts are represented in proportional to the complexity of the movements that they perform. The body parts that make the finest movements like hand and face occupy larger areas compared with those for the rest of the body. The motor homunculus is only a sketch proportional representation of different body parts on the motor cortex as there is high overlapping of adjacent body parts. The upright face representation in the homunculus is also incorrect. It was until late 20th century when it was discovered that, the face representation in inverted (Servos et al., 1999). Servos and colleagues (1999) using functional Magnetic Resonance Image (fMRI) technique to study activity of
neurologically intact human motor cortex during face stimulation showed that; when the chin
was stimulated the cortical activity increased in the superior site of the post-central gyrus near
hand area, while forehead stimulation was associated with increased activity far inferior to the
hand representation area.

Penfield’s experiments found an overlapping of sensory and motor functions in the
cortex, although movements were typically evoked more anterior to the central fissure and
sensations were typically evoked more posterior to the fissure (Penfield and Rasmussen, 1968).
It was found that, about 80 percent of the motor responses are evoked in the precentral cortex
and 20 percent in the postcentral cortex. It was further noted that evoked movements within the
post-central gyrus were independent of the precentral gyrus, as they were produced even after
removal of the adjacent precentral cortical region (Penfield and Rasmussen, 1968). In other
words, the motor cortex is not restricted in the precentral gyrus; it extends to what is traditionally
called somatosensory cortex. The similar topographic representation of body parts on the
precentral gyrus described by Penfield and colleagues does exist in other mammalian species as
well. Experiments in human and monkey brain (Penfield and Welch, 1951) discovered a second
motor area located on the superior and medial aspect of the cerebral hemisphere; the SMA,
which involves in planning complex movements and in coordinating movements which
sometimes involve both sides of the body. Penfield and his colleagues’ motor maps were shortly
confirmed by Woolsey et al. (1951), who named the monkey motor map, a simiusculi.
Interestingly, the movement representations in the SMA map is more highly overlapping than in
the lateral map (Gould et al., 1986; Luppino et al., 1991; Woolsey et al., 1951).
Studies of changes in regional cerebral blood flow (rCBF) in people gave evidence of a distinction between the primary motor cortex, premotor cortex, and SMA (Roland et al., 1980). Roland and colleagues used fMRI technique to measure rCBF while participants performed various tasks. Their studies indicated that SMA is involved in the execution of complex movement sequences and indeed in the programming of such sequences, since the SMA exclusively showed an increase in rCBF when subjects were asked to think about the movements without executing them (Roland et al., 1980). When persons executed simple movement like manually palpating an object, rCBF increased to the primary motor cortex (Roland et al., 1980).

Additionally, execution of complex movements increased rCBF to the both SMAs, and in the contralateral primary motor and sensory areas. This indicates that complex movements are controlled by bilateral SMAs (Roland et al., 1980). rCBF increased to the premotor cortex when persons performed new tasks on the basis of ongoing instructions (Roland et al., 1980). A few years later, Cobie Brinkman confirmed Roland and colleagues’ suggestions that the SMA is involved in programming and coordinating complex movements. In his experiment, he found that SMA lesioned monkeys and not premotor lesioned ones showed a characteristic deficit of bimanual coordination (Brinkman, 1984). The SMA also plays a role in coordinating posture and voluntary movements (Viallet et al., 1992). There is also premotor area located within the cingulate gyrus, important in allowing motivation to influence motor planning directly (Luppino et al., 1991). As movements are represented in the primary motor cortex and SMA, it is also suggested that such representation exists in the premotor cortex (Dum and Strick, 2005; Rizzolatti et al., 1988).
Scientists also divided the motor cortex into regions on the basis of cell appearance and fiber tracts. Alfred Campbell and Korbinian Brodmann divided the motor cortex into two cytoarchitectonic regions, posterior and anterior (Campbell, 1905; Strotzer, 2009). Campbell (1905) defined 17 cytoarchitectonic cortical areas labelled by functions. In this division, he divided the motor cortex into precentral and intermediate precentral cortices. Campbell’s posterior region (precentral gyrus) is characterized by dense population of giant pyramidal cells (Betz cells) in the deeper layers and the anterior region (intermediate precentral cortex) lacks Betz cells. The two regions lack a clear demarcation. Campbell (1905) suggested that the precentral cortex has direct control of muscles, and termed it primary motor cortex as it was earlier called by Beevor and Horsley. Campbell speculated that, the intermediate precentral cortex is the highest level of control of complex coordinated movements.

In 1909, the neurologist; Korbinian Brodmann defined 52 separate areas of the cerebral cortex based on cytoarchitectonic features (Strotzer, 2009). He studied histology of brains of small and large mammals including human using the Nissl (nucleic acid) staining method. Brodmann’s divisions were based on cell composition of different cortical layers, cell density, cell size and cortical thickness. Cortical regions with similar cellular and laminar structure were labelled the same number, Brodmann’s area 1 - 52. The boundaries between the Brodmann’s areas varied. In some areas the borders are sharp, while in other area they borders are poorly defined (Strotzer, 2009). Furthermore, Brodmann felt that these anatomically singular areas were also distinct functionally (Strotzer, 2009). The motor cortex as it is understood today occupies Brodmann’s areas 4 (localized in the precentral gyrus), 6 (agranular frontal area), 44 (in the inferior frontal gyrus) and areas 1, 2 and 3 in the postcentral region. However, it should be clear
that the cerebral cortex cytoarchitectonic studies, began even before Brodmann was born. It was already known that, the cerebral cortex is made up of six layers, which were given different names by different scientists.

In 1919, Cecile Vogt and Oskar Vogt extended Brodmann’s work by describing more different cortical regions. Cecile Vogt and Oskar Vogt also divided the motor cortex into anterior and posterior region on basis of histological appearance. They stimulated these motor areas and found that, low electric current stimulation of both anterior and posterior regions evoke simple movements of separate body parts, and stimulation of anterior region at higher currents produce complex movements that combine more than one body part (Graziano, 2009). They further suggested that, the anterior region (Brodmann area 6) controls both simple movements through lateral projections to area 4, and complex movements through deep projections probably directly to the spinal cord or to subcortical nuclei. This followed their experiments of transacting anterior region longitudinally and horizontally (Graziano, 2009).

The overlapping of movement representations in the motor cortex observed by Penfield and colleagues was suspected to be caused by spread of electrical currents over the cortical surface. The development of intracortical microstimulation (ICMS) technique that is more precise in stimulating areas of the cerebral cortex confirmed that there is overlap of the body parts represented on the motor cortex. Hiroshi Asanuma and colleagues are honored for developing this precise stimulation method, though their experiments using cats wrongly suggested that, each muscle is separately represented in the motor cortex. Their suggestion was proved wrong by studies which recorded single cortical neuron activity (Cheney and Fetz, 1985; Cheney et al., 1985; Schieber and Hibbard, 1993). The use of stimulus triggered activities of
individual primary motor cortex neurons in monkeys clearly showed that no individual muscle representation exists as was suggested by Asanuma and colleagues (Cheney and Fetz, 1985; Cheney et al., 1985). Cheney and colleagues demonstrated that, each corticomotorneuron (CMN) innervates spinal motoneurons that innervate a set of muscles and each muscle is innervated by many CMNs. Stimulation of single CMNs causes the facilitation and inhibition in a varying combination (Cheney and Fetz, 1985; Cheney et al., 1985). Schieber and Hibbard (1993) supported the fact that, muscles are not represented discretely in the motor cortex. Schieber and Hibbard trained monkeys to make extension and flexion of individual fingers, and later observed single neuron activity in the hand representation as the monkey performs various tasks. They noted that, majority of neurons fire following movement of more than one finger. Microstimulation of monkey brain was extended to involve other forelimb movement areas apart from fingers.

Many other studies also showed an extensive overlapped representation of forelimb muscles in the motor cortex (Donoghue et al., 1992; Gould et al., 1986; Kwan et al., 1978; Park et al., 2004; Park et al., 2001). Furthermore it was found that this overlap tends to increase with behavioral experience (Martin et al., 2005; Nudo et al., 1996). Martin and colleagues (2005) using cats demonstrated that, at birth the separate joints of the forelimb are represented in the motor cortex in discrete patches, but with development of complex behaviours the forelimb joints representation becomes overlapped. Overlapping forelimb joint cortical representation as a result of increased forelimb activity were also observed in experiments using monkeys (Nudo et al., 1996).
Finally the famous French neurologist Broca also contributed to our understanding of the motor cortex for his discovery of the speech production center (Broca, 1861), now known as Broca's area. Broca’s area is not only for the memory of words, but also controls word articulation. Thus, Broca’s region controls mouth and tongue movements. He arrived at this discovery in 1861 by studying the brain of Leborgne, who was aphasic. The patient was given a nicknamed "Tan" due to his inability to clearly speak any words other than "tan". Later, the patient developed weaknesses of the right arm and leg followed by paralysis. Autopsy found a left frontal lobe lesion. Broca (1861) suggested that, the lesion begun in the speech area and later spread to affect limb control centers in the precentral gyrus causing weakness and paralysis of right limbs.

In brief, we have seen that cortical sites anterior and posterior to the central sulcus control the contralateral body parts movements. These sites constitute the motor cortex and they have neurons which send their axons directly to the spinal cord. The body parts movements are topographically represented in the motor cortex in an upside manner, and such representations are not discrete. The subsequent sections discuss how this representation is altered by seizures.

1.5 Seizures, Epilepsy and Motor Maps

1. 5.1 Motor Maps

As detailed earlier in this review, motor maps refer to a topographic representation of body part movements in different areas of the motor cortex. Many different stimulation techniques are used to evoke movement during motor mapping. These techniques differ in the type of stimulation used to evoke movement as well as how the motor responses are measured.
In human and other large animals, the motor maps are derived by subdural electrical or transcranial magnetic stimulations (TMS) of different points of the motor cortex and observing the activity of individual muscles. Subdural electrical stimulation is performed by application of electric current on the cortical surface through the electrodes which are surgically implanted under the dura matter. The TMS works by inducing an electric field in the brain. For both of these techniques, the output is usually measured by changes in the activity of individual muscles measured by electromyography (EMG).

In small animals like rats and mice, the motor maps are obtained by stimulating different areas of the motor cortex using microelectrodes and observing the evoked movements. ICMS provides the fine details of the motor map because it uses much less current (1/100th of surface stimulation) and is therefore more focal and gives high resolution maps.

Positron emission tomography (PET) and fMRI have also been used to study activation maps of the motor cortex. The PET and fMRI techniques are performed to map normal human brain function, among other purposes. In the PET technique, the radiotracer injected into a vein which eventually accumulates in the brain and gives off gamma rays which are detected by a gamma camera, a PET scanner and/or probe. The amount of radiotracer absorbed by the brain produces pictures offering details on neuronal activity or regional blood flow. Brain neural activity causes changes in local blood flow and blood oxygenation. fMRI is used to detect the localized changes in blood flow and blood oxygenation that occur in the brain in response to neural activity. Measurement of neuronal activity and changes in regional blood flow and oxygenation during particular body movements enables us to localize the body parts in the motor cortex. Human and animal studies employing the above describe methods have demonstrated
that the organization of motor maps are not fixed, they vary under circumstances. The next two sections (1.5.2 – 1.5.3) describe the motor map reorganization that occurs with seizures.

1. 5.2 Effects of Epilepsy on Motor Maps

Seizures that affect the motor areas of the brain have the potential to disrupt the functioning of these structures both during and between seizures. The study of motor cortex in patients with epilepsy has a long history. As pointed out earlier, eighty years before Wilder Penfield, and later Woolsey and others described the motor maps in detail, the neurologist John Hughlings Jackson had suggested that the motor maps of brains of patients with epilepsy tend to reorganize. Much of the earlier studies of motor mapping came from surface stimulation experiments conducted before surgery to excise epileptic foci.

Studies involving patients with epilepsy have demonstrated that repeated epileptic seizures results into motor maps expansion (Lado et al., 2002b; Lee et al., 2009; Uematsu et al., 1992; Urasaki et al., 1994). For instance, in 1992, chronic subdural electrode stimulation showed that epilepsy causes the motor area to be detected more anterior and posterior to the central sulcus/fissure. The primary motor cortex which lies within the narrow strip of (within 10 mm) the precentral gyrus was found to lie beyond this area, extending the area of movement representations anterior and posterior following seizures (Uematsu et al., 1992). The same authors observed that motor maps expanded more in epileptic patients with a brain lesion, compared to those without the lesion. Urasaki and colleagues (1994), using subdural electrical stimulation observed expansion of a tongue representation in the motor cortex. The tongue sensorimotor area was found to take 4.5 cm anterior to and 3 cm posterior to the central sulcus (Urasaki et al., 1994), while in human brain the tongue area occupies only about 1.5 cm mostly
anterior to the central sulcus. In the same study, the tongue area was observed to be unusually extended several centimetres medially in epileptic patients (Urasaki et al., 1994). Other studies using subdural electrical stimulation on epileptic patients reported the variations of cortical motor representation over time. Cortical motor mapping at two different occasions noted face (Lado et al., 2002b; Lee et al., 2009), tongue, hand, finger or leg (Lee et al., 2009) response in areas which showed no motor response during the first procedure. Lado et al. (2002) found a left facial somatotopic representation which was located on the right precentral gyrus of an epilepsy partialis continua patient to be displaced towards the vertex five years later and the left arm and eye cortical motor area were also abnormally organized at the same interval of five years.

Epilepsy, not only expands the motor maps but also results in enhanced overlap of different body parts representations on the primary motor cortex (mosaicism), evidenced by multiple movements following electrical stimulation of one point in the primary motor cortex (Branco et al., 2003). The same authors reported that, there is an overlap of sensory and motor centers in uninjured brain.

Studies employing PET and fMRI in patients with epilepsy have also shown reorganization of the motor maps, evidenced by a bilateral activation of the sensorimotor cortices during movement of the hand on opposite side of the epileptic lobe (Chlebus et al., 2004; Stoeckel et al., 2002), and subsequent shift of handedness (Chlebus et al., 2004). It was also noted that bilateral activations manifested well during finger movements (Stoeckel et al., 2002). Resting brain activity in patients with epilepsy as measured by fMRI, showed significant reduction of motor network connectivity between the left and right sensorimotor cortices, and that this reduction is directly proportion to seizures frequency (Woodward et al., 2014a). Furthermore, fMRI studies in patients with frontal lobe epilepsy demonstrated decreased brain
activity in the epileptic hemisphere and increased brain activity in the healthy hemisphere, the changes which tend to decrease with time after seizures are controlled (Woodward et al., 2014b). Reorganization of motor network observed in these studies suggests that motor network disturbances are likely causes of the motor deficits which occur in patients with epilepsy (Woodward and Federico, 2014).

The use of TMS technique confirmed the reorganization of motor cortex in patients with focal motor seizures (Hamer et al., 2005; Labyt et al., 2007). The studies using TMS have also demonstrated decreased cortical stimulation period (CSP) in the ipsilateral epileptic motor cortex (Hamer et al., 2005), higher resting motor threshold and an asymmetrical interhemispheric abductor pollicis brevis (APB) muscle representations in epileptic patients (Labyt et al., 2007). Shortening of CSP was noted to vary with type of epilepsy, with extratemporal epilepsies producing much reduction (Hamer et al., 2005).

1.5.3 Laboratory Animal Studies on Motor Maps

There is a pressing need to carry out extensive experiments to improve the epilepsy diagnostic, treatment and preventive methods. The best subjects for studying the mechanisms underlying epilepsy and plan for management of this neurological disorder are patients in epilepsy surgery facilities (Engel, 1998). However, there are a number of unavoidable issues that limit the use of patients with epilepsy in research. These restrictions include ethical considerations especially on use of invasive procedures and patient’s rights, stress and stigma, interpersonal variability and the use of different drugs in the clinical setting and imprecision in defining epileptic focus. Other limitations are difficulty in data control due to the distributed nature of the epileptogenic abnormalities and other disturbances; and small number of subjects which makes it difficult to validate the statistical significance (Engel, 1998). Therefore animal
models of seizures and epilepsy are largely used to expand our understanding of neuronal mechanisms of the normal brain function and investigate fundamental mechanisms of abnormal electrical discharge and understand the pathogenesis of epilepsy (Engel, 2001). Although it is unlikely that any animal model will precisely mimic all aspects of epilepsy, it is important tool to identify cellular and molecular mechanisms that might be involved to cause epileptic seizures and its consequences (Engel, 1998). Further, animal models of epilepsy are also necessary for researches which are designed to devise and improve diagnostic techniques and in the development of antiepileptic drugs (Lösch, 1997; Lösch, 2002; Lösch and Schmidt, 1988).

There are several seizure models. Kindling, which was discovered by Graham Goddard in the late 1960s is one example (Goddard, 1967). Kindling refers to a technique whereby seizures repeatedly elicited over time results in epileptogenesis. Epileptogenesis is the state of increased susceptibility of seizure occurrence, thus epileptogenesis is associated with lowered seizure threshold. In kindling repeatedly induced seizures results in increasing seizure duration and enhanced behavioral involvement of those induced seizures. Therefore, kindling also refers to a phenomenon in which the afterdischarges (AD) and seizures become more prolonged and severe with increased stimulation sessions (Corcoran and Teskey, 2009; Goddard, 1967; Goddard et al., 1969; McIntyre and Gilby, 2009; Racine, 1972b).

Goddard (1967) discovered kindling accidentally while examining the effects of electrical stimulation of the amygdala on avoidance conditioning in rats. During his experiments one of the rats was observed to develop behavioural seizures following repeated electrical stimulation of the amygdale while other rats who did not have repeated stimulations did not develop seizures. This led Goddard to conclude that seizures occurred as a result of repeated electrical stimulation and not due to stimulation per se. Goddard further deduced that repeated electrical stimulation caused
changes in brain structure leading to increased susceptibility to seizure occurrence, since at first
the stimulation had no effect on behavior and did not cause electrographic seizures but with
repeated stimulation the rat developed localized seizures which progressively worsened to full
blown convulsions. Goddard called this phenomenon ‘kindling”, the term which was accepted
and being used universally. Goddard’s speculations were correct, indeed a number of structural
and chemical changes occur during kindling.

For kindling to occur, stimulation must elicit AD (Goddard et al., 1969; Racine, 1972b;
Racine, 1972a). Repeated stimulation can be done by chemical convulsant drugs (eg cholinergic
like pilorcapine or anti-GABAergic drug like bicuculine) or electrical. Of the two ways of
kindling, electrical stimulation is the preferred method because it is more precise. You can
reliably stimulate the brain region of interest. Electrical kindling is done by applying brief trains
of pulses, typically 1 or at most 2 seconds in duration into brain area of interest (such as corpus
callosum, amygdala or hippocampus) through surgically implanted wire electrodes (Racine,
1972b; Racine, 1972a). Stimulation consisting of 1 second train with 60 Hz is used most
frequently. However, 120 second trains of 3 Hz waveforms have also been used to induce
seizures. The waveform is usually balanced biphasic square pulses, although some experimenters
apply monophasic (cathodal) pulses. The intensity of stimulation is set above the threshold for
generating afterdischarge. EEG is recorded before, during and after stimulation. Electrical
stimulation induces hypersynchronous neuronal discharges, which spread to all areas of the brain
and spinal cord and thus drive the behavioural seizure. Electrical stimulation at first produces
brief afterdischarge without or with minimal behavioural manifestation. With repeated
stimulations there is a progressive increase in afterdischarge duration (ADD) and development
and worsening of behavioural manifestation of seizure (Corcoran and Teskey, 2009; Goddard,
1967; Goddard et al., 1969; McIntyre and Gilby, 2009; Racine, 1972b). Seizures can reliably be kindled when stimulations are sufficiently spaced. Racine and colleagues (1972b) found that AD is reliably elicited if repeated stimulations are applied at the intervals of at least 2 hours, although some studies have shown that it is possible to elicit AD at shorter interval than 2 hours. Stimulation at shorter intervals leads to short-lasting inhibitory effects that can suppress AD or the behavioral seizures triggered by AD.

Kindling also lowers afterdischarge threshold (ADT) (Racine, 1972a), thus increasing susceptibility of seizure occurrence and with prolonged repeated stimulations, spontaneous seizures may occur (Brandt et al., 2004; Michalakis et al., 1998; Pinel et al., 1975; Wada and Osawa, 1976). Reduction in threshold for AD following kindling persists for long time if not permanent (Goddard et al., 1969; Hiyoshi and Wada, 1992; Racine, 1972a). Reduced ADT and increasing ADD during kindling is due to physiological, neurochemical and morphological changes at synapses. Kindling is associated with increased synaptic potentiation (Racine et al., 1991) as well as reduced GABAergic inhibition (Leung et al., 2005; Shinnick-Gallagher et al., 1998). Racine and colleagues (1991) were the first to report potentiation of potentials evoked in secondary sites during kindling stimulation. In their studies, they found that kindling of one limbic or cortical site results in a facilitation of subsequent kindling from other limbic or cortical sites (Racine et al., 1991), suggesting that seizures activity during kindling spread and affect other brain areas. Kindling induced potentiation is associated with long lasting increase in glutamate release (Jarvie et al., 1990). Neurotrophins are thought to play a critical role in development of epileptogenesis (He et al., 2004). Major structural change associated with kindling is increased proportion of highly efficacious perforated synapses (Geinisman et al., 1992; Henry et al., 2008) which are thought to be responsible for maintaining kindling induced
potentiation. Perforated synapses have narrower synaptic cleft between presynaptic and postsynaptic membranes at the site of the perforation and have greater glutamate receptors on postsynaptic membranes than non-perforated synapses.

Kindling, after first been demonstrated in rats (Goddard, 1967), it was also found to occur in cats (Goddard and Morrell, 1971), monkeys (Goddard et al., 1969), rabbits (Tanaka, 1972) and mice (Leech, 1972). Human brain also does kindle. Evidence that human brain can be kindled is development of epileptogenesis which has been reported to occur in human following repeated deep brain stimulation during electroconvulsive therapy in managing resistant depression (Rasmussen and Lunde, 2007) and chronic pain (Šramka et al., 1977). Deep brain stimulation is done after surgical implantation of electrodes into specific areas of the brain, and then electrically stimulating these areas at prescribed time interval. Furthermore, in clinical practice it has been observed that, occurrence of one seizure increases the risk for a subsequent seizure (Graves et al., 2012). For example repeated or prolonged febrile convulsions increase the risk of epilepsy (Annegers et al., 1979). Therefore it is thought that each seizure lowers the threshold for a subsequent seizure, suggesting that seizures cause changes in neuronal networks or neurotransmitter release which lead to increased neuronal excitability. Increased risk for subsequent seizure (epileptogenesis) in human suggests that human brain kindles. Another suggestion that human brain can kindle is the observation that repeated seizures in people with epilepsy leads to prolonged seizure duration and development and worsening of behavioural manifestation. Many patients who developed focal seizures with impaired consciousness or awareness had been having history of subjective feeling or psychic phenomenon which gets prolonged before onset of clinical seizures (Glaser, 1987). Other patients history indicate to have focal seizures which tend to worsen with time evidenced with more severe motor manifestations.
which sometimes lead to injury. Generally, these patient histories suggest progressive increase in seizure duration and worsening of behavioural manifestations which is the characteristic of kindling.

For more than 45 years, since it was first discovered, kindling has been used as a model for human temporal lobe epilepsy and for epileptogenesis by many investigators. There are many similarities between kindling and human epilepsy, especially temporal lobe epilepsy, making kindling a good model of epilepsy (McIntyre and Gilby, 2009). These similarities include the behavioural patterns which occur and the EEG activity recorded during seizure. Other similarities include the development of inter-ictal spikes, effectiveness of drugs which can arrest seizures and possibility of occurrence of seizures, which is the main feature defining epilepsy. It is easier to study the effects of seizures on cortical motor map expression because in kindled animals the occurrence of seizures is not only predictable, but the process is also highly reliable. However, there are some differences between kindling and human epilepsy. For instance, the likelihood of occurrence of unprovoked seizures is greater in patients with epilepsy than in kindled animals. Mechanisms of development of epileptogenesis also differ greatly. Structural or metabolic derangements and genetic malformations are the major aetiological factors for epilepsy while kindling is derived only through repeated brain stimulation. The other difference is morphological changes which occur in the two conditions. There is relatively defined pattern of neuronal loss and glial hypertrophy in epileptic foci (Bertmam and Lothman, 1993; Bertram et al., 1990; Mathern et al., 1997) but these structural changes are not so pronounced in kindled brain areas (Adams et al., 1998; Cavazos et al., 1994; Mathern et al., 1997).
The laboratory of Dr. Cam Teskey has found that the repeated induction of seizures in rats results in larger motor maps, lower movement thresholds and more multiple movements (Henderson et al., 2011; Teskey et al., 2002; van Rooyen et al., 2006; Young et al., 2011b; Young et al., 2009; Ozen et al., 2008). Teskey and colleagues (2002) were the first to report doubling of the motor map size, after observing the caudal forelimb motor area in kindled adult rats’ motor cortex being approximately twice as large as control rats. Increased forelimb area representation in experimentally induced seizure rats is now known to be associated with disturbance of skilled forelimb movements (Henry et al., 2008), the cause of disruption of skilled forelimb behaviour being the frequent seizures, and not other neuronal electrical induced alteration such as lowered ADTs (Flynn et al., 2010). Additionally, it has been shown that, at higher ICMS intensities, kindled rats produce multiple forelimb muscle responses at many points of the motor cortex, compared to non-kindled rats (Teskey et al., 2002).

Reorganization of the motor map by seizures has been shown to be persist up to 5 weeks without loss of size (Ozen et al., 2008; Teskey et al., 2002) and linearly related to the number of seizures that propagated to the motor cortex from the hippocampus (van Rooyen et al., 2006). Thus, the higher the number of the cortical seizures leads to the bigger the motor maps. More recent findings show that motor map expansions in kindled rats are proportional to the severity of the seizures induced by the chemical convulsant pilocarpine (Young et al., 2009).

The increased motor maps are associated with enhanced horizontal fibers synaptic potentiation (Henry et al., 2008; Teskey, 2009). High frequency stimulation of the corpus callosum induces horizontal fibers potentiation leading to increased motor maps size (Henry et al., 2008) regardless of whether seizures were evoked or not (Teskey, 2009). The opposite is
true; low frequency stimulation induce horizontal fibers depression and causing a reduction in the caudal forelimb representation in the motor cortex (Teskey et al., 2007), and repeated low frequency stimulations are associated with a reversal of motor map expansion following experimentally induced seizures (Ozen and Teskey, 2009; Ozen et al., 2008). The decrease in motor map size following low frequency stimulation does not affect the neocortical thickness or number of neurons (Henry et al., 2008). A reduction of the excitatory perforated synapses in the layer V of the sensorimotor neocortex following low frequency stimulation has been observed (Teskey et al., 2007). The long-term potentiation in the motor neocortex which expands movement representations has been shown to be due a loss of GABAergic inhibition (Jacobs and Donoghue, 1991), and enhancement of glutamatergic transmission (van Rooyen et al., 2006).

Genetic predisposition is also thought to affect motor map expansion (Young et al., 2009), as strain differences in seizure-induced motor map expansion has been demonstrated. Experiments done using cats showed that seizures also change sensory cortical maps (Vuong et al., 2011; Teskey, 2009).

To sum up we can say that motor maps are cortical networks which when stimulated elicits activity of specific group of muscles which may bring a particular body movement depending on stimulus strength. The cortical networks which constituent motor maps, contain, in layer V, efferent neurons which project either to the spinal cord or brain stem. They also contain interneurons and neurons which project to adjacent cortical areas. Thus, there are interconnections of fibers within the motor map and between the motor maps and other cortical areas. These cortical networks are responsible for initiating voluntary or planned movements of contralateral side of the body. When a particular point in the motor map is stimulated, specific
muscles in the body will contract. Thus, voluntary muscles of the body are topographically represented in the contralateral motor cortex, but the muscles of the body axis are bilaterally represented. Body parts representation in the motor cortex is more complex than the arrangement pictured in Penfield’s motor homunculus. Representation of body movement parts is not discrete but there is an overlapping of body parts. The proportion of representation depends on complexity of activity performed by a particular body segment. Signals for movement travel through corticobulbar or corticospinal tracts. Corticobulbar tract neurons activate cranial neurons which control muscles of facial expression while corticospinal tract neurons activate spinal cord motor neurons which control limb and trunk muscles.

The organization of motor maps are not fixed, rather they vary under circumstances. Experience and behaviour alter motor maps. For instance, increased skilled movements lead to motor maps expansion and increased overlapping of body representation. Seizures also cause reorganisation of motor maps network, they increase motor map sizes and mosaicism and lower movement thresholds. In addition to alteration of the motor maps, seizures are now known to induce severe and long lasting local brain hypoxia. This recently discovered phenomenon is described in the next section.

1.6 Seizure Induced Severe Hypoxic Episodes (SISHE)

Seizures are associated with changes in brain metabolic rate (MR) (Blennow et al., 1977; Howse et al., 1974; Meldrum and Nilsson, 1976) and cerebral blood flow (CBF). Whole brain metabolic rate tend to increase up to between 200% and 300% during seizures (Meldrum and Nilsson, 1976). Status epilepticus causes a 6 to 10 times increase in the brain MR at the beginning of the ictal phase (Blennow et al., 1977; Howse et al., 1974), however the MR later
falls to almost zero inspite of ongoing status epilepticus (Howse et al., 1974; Ingvar and Siesjo, 1983). Interictal increase in CBF has been observed in both animals and man experiments (Hougaard et al., 1976; Meldrum and Nilsson, 1976; Penfield et al., 1939; Schmidt et al., 1945). Penfield et al. (1939) were the first to make this observation and speculated that this change was a consequence of increased neuronal activity. Increased CBF following generalized seizures has been reported to be of magnitude of up to 900 % as compared to controls (Meldrum and Nilsson, 1976; Schmidt et al., 1945). The increase in cerebral blood flow is very rapid and detectable within seconds after the onset of a generalized epileptic seizure (White et al., 1961).

Despite the known changes in brain metabolism and CBF during ictal and post-ictal phases of seizures, until 2012 there has been limited information regarding changes in brain oxygen levels during and after seizures. Although changes in brain oxygen levels during and post-seizures were unknown, it was known that the short- and long-term effects of seizures are more similar to effects of brain ischemia/hypoxia. For instance, it has long been known that seizures cause brain damage (Kalviainen et al., 1998; Lado et al., 2002a; Holmes, 2002; Holmes et al., 2002; Liu et al., 2005; Kotloski et al., 2002), the effect which is also caused by brain hypoxia/ischemia (Gale and Hopkins, 2004; Malhotra et al., 2001; Mattiesen et al., 2009; Vintila et al., 2010). Furthermore, both seizures and brain ischemia frequently lead to either transient or permanent impairment of sensory, cognitive or motor functioning (Yan et al., 2011; Vintila et al., 2010; Davies et al., 2013; Ibrahim et al., 2012; Smith et al., 2002; Hernandez et al., 2003; Sanchez-Carpintero and Neville, 2003; Oddo et al., 2003).

Teskey and Farrell (2012) speculated that a seizure may lead to a cascade of events, which result in vasoconstriction and an ischemic/hypoxic episode. Using a chronically implanted oxygen-sensing device, Teskey and Farrell (2012) observed that there is severe and long-lasting
hypoxic episode in brain tissues, which begins after seizures terminate. They termed this event seizure induced severe hypoxic episode (SISHE). Demonstration of SISHE is shown in figure 1-4 below which was the record taken from the rat hippocampus.

In their experiments, Teskey and Farrell (2012) were able to ascertain that SISHE is due to vasoconstriction as vasodilator drugs and agents that block vasoconstriction, without altering the duration or severity of the seizure itself, prevented it. Seizures induced vasoconstriction is likely due to initiation of series of events that occur over progressively longer temporal scales and involve numerous cell types including neurons, astrocytes, pericytes, smooth muscle, and endothelial cells as well as a number of ions, ion channels, neurotransmitters, other signaling molecules, enzymes and contractile proteins. The cascade of reaction are thought to result into abolishment of the cerebral blood flow autoregulatory mechanisms leading to abnormal levels and long durations of vascular constriction, leading to local tissue hypoxia.

Teskey and Farrell (2012) observed seizure-induced hypoxia in the hippocampus, amygdala, and neocortex when kindled seizures are elicited within those structures. Thus, seizure-induced hypoxia is a local event as it is only observed in brain areas that are involved in the epileptiform discharge, and it is not related to changes cardiac output or systemic blood pressure. When epileptiform activity elicited in the amygdala propagated to the motor cortex, seizure-induced hypoxia is then, and only then, observed in the motor cortex.
Figure 1-4: Seizure induced severe hypoxic episode

Figure 1-4 shows a long lasting severe hypoxia (pO₂ < 10 mmHg) post-seizure. The upper panel shows 1 second of kindling stimulation applied at time point (a) and the resulting electrographic seizure which terminates at time point (b). In the lower panel the mean partial pressure of oxygen (pO₂ mmHg) in the rat dorsal hippocampus from an indwelling optrode. Time during the electrographic seizures is again indicated by (a) and (b) and time below the severe hypoxic threshold (<10 pO₂ mmHg) is indicated between (c) and (d).
Teskey and Farrell (2012) also confirmed that localized significantly reduced blood perfusion accompanies this hypoxic period. Changes of regional cerebral blood flow and brain oxygen levels post-seizures are shown in figure 1-5 below. Collaboration with Dr. Paolo Federico (unpublished) has revealed this phenomenon also occurs in human patients implanted with an oxygen sensing device. Therefore, seizure induced sensory, cognitive and behavioral dysfunctions are likely to be a result of brain ischemia/hypoxia in addition to other mechanisms. Despite the fact that seizures disrupt blood flow and metabolism, oxygen levels are the most important determining factor of the brain neuronal dysfunction. When blood supply to the brain tissue is severely decreased and cannot keep up with metabolic demand, brain cells intracellular oxygen and glucose depletion occurs. When brain tissue oxygen levels fall below the severe hypoxic threshold ($pO_2 < 10$ mmHg), cellular dysfunction with short and long term neurological and behavioural consequences result. Farrell and Teskey (unpublished data) demonstrated that neocortical seizure hypoxia made rats to perform more poorly on the hanging bar task and this deficit in forelimb strength was abolished by agents that prevent SISHE.

Furthermore, Farrell and Teskey (unpublished data) have shown that hippocampal SISHE leads to a period of anterograde amnesia in rats. This suggests that many of the behavioural deficits that follow seizures may be explained by SISHE and not the seizure per se. Thus SISHE is likely to be responsible for Todd’s Paresis, occurring post-seizure.
Figure 1-5: Seizure induced severe hypoxia and reduced local cerebral blood flow

Figure 1-5: Change in blood flow and pO$_2$ in the hippocampus after a seizure. SISHE is associated with a reduction in local blood perfusion.
1.7 Thesis Objectives and Hypothesis

Since seizures alter motor functioning and induce severe local brain hypoxia and ASDs control them, these drugs were therefore speculated to have effects on the expression of the motor maps and seizure induced severe ischemic hypoxic episodes. Due to the interconnected networks of neurons, particularly the local horizontal connections of layer V pyramidal neurons that make up the corticospinal tracts, it was hypothesized that **anti-seizure drugs would directly alter motor maps expression and prevent the development of seizure induced severe ischemic hypoxic episodes or reduce its severity**. In chapter two, I demonstrate the effects of classic ASDs on motor map expression while in chapter three I demonstrate the inhibitory effects of the putative ASD, bumetanide on brain activity. Furthermore, in chapter four I describe the effects of various ASDs on seizure induced severe hypoxic episode. The three chapters altogether share the common theme to address the effects of anti-seizure drugs on neuronal networks (motor maps) and neurovascular function.

The aim of chapter two was to determine the effects of classic ASDs on motor maps expression. To accomplish this aim, standard ICMSs were performed in anesthetized rats where motor maps were derived by recording movement types and their thresholds. This was followed by intraperitoneal (i.p) injection of ASDs or vehicles and re-deriving the maps after 30 minutes (Biggs et al., 1992; Gilbert et al., 2001; Gower et al., 1995). Post-ASDs or vehicles movement thresholds, and forelimb area sizes were then compared to the baseline values. **It was hypothesized that ASDs would raise movement thresholds and cause reduction of the size of forelimb area of representations in the motor cortex which respond to electrical stimulation.**
The objective of chapter three was to determine whether bumetanide inhibits adult brain neurons and the effects of this drug on motor maps expression. To accomplish this aim, kindling of young adult rats was done for 15 days. On days 2, 5 and 15 bumetanide or saline was given 30 minutes before seizure induction (Mareš, 2009; Mazarati et al., 2009). Afterdischarge thresholds (ADTs), afterdischarge durations (ADDs) and seizure stages were then determined. Post-bumetanide ADTs, ADDs and seizure stages were then compared to values obtained from saline control rats. Additionally, standard ICMSs (as in chapter two) were performed in young adult naïve and 3Hz-kindled rats. Post-bumetanide or saline movement thresholds, and forelimb area sizes were compared to the baseline values. Furthermore, electrophysiological studies to determine the effect of bumetanide on layer V pyramidal neurons were done. It was hypothesized that bumetanide would raise ADTs, reduce ADDs and seizure severity as well as elevating movement thresholds, inhibiting layer V pyramidal neurons and reduce the size of forelimb area of representations in the motor cortex.

The objective of chapter four was to determine the effects of ASDs and bumetanide on seizure induced severe hypoxic episode. To achieve this aim both an electrode and an oxygen sensing device were implanted into the dorsal hippocampus (CA1). After a week recovery period, I recorded baseline O₂ levels and then induced seizures in rats to obtain post seizure O₂ levels. Rats who developed SISHE (hypocampal O₂ level of less than 10 mmHg) post seizures were tested by inducing seizures 30 minutes after i.p injection of ASDs or bumetanide or their vehicles and measuring post-seizure hypocampal O₂ levels. No drug post-seizure O₂ levels, post-vehicle and post-drugs O₂ levels were compared. It was hypothesized that ASDs and bumetanide would prevent development of SISHE or decrease its severity.
CHAPTER TWO

EFFECTS OF CLASSIC ANTI-SEIZURE DRUGS ON MOTOR MAPS EXPRESSION

2.1 Introduction

Motor maps are topographic representation of body part movements in different areas of the motor cortex. In mammals this representation is in cortical regions anterior and posterior to the central sulcus (Beevor and Horsley, 1887; Ferrier, 1873; Ferrier, 1874; Grunbaum and Sherrington, 1901; Grunbaum and Sherrington, 1903; Penfield and Rasmussen, 1968). Here the body representation is organized upside down on the contralateral cerebral hemisphere (Ferrier, 1873; Ferrier, 1874; Penfield and Rasmussen, 1968). The different body parts representation in the motor cortex is not identical but depends on the complexity of the movements that they perform. The body parts that make the finest movements like hand and face occupy larger areas than other body parts (Penfield and Rasmussen, 1968). In human and other large animals, the motor maps are derived by subdural electrical or transcranial magnetic stimulations of different points of the motor cortex and observing the changes in activity of individual muscles measured by electromyography. Positron Emission Tomography (PET) and functional Magnetic Resonance Imaging (fMRI) are other techniques which may be used to study motor maps in large animals. In small animals like rats the expression of motor maps are studied by intracortical microstimulation (ICMS) where the observed output is evoked movements.

Human and animal studies have shown that seizures and epilepsy alter motor maps expression. Expansions of motor maps have been observed in patients with epilepsy (Lado et al., 2002b; Lee et al., 2009; Uematsu et al., 1992; Urasaki et al., 1994). Seizures in patients with epilepsy, not only expand the motor maps but also increase overlapping of different body parts
representations (Branco et al., 2003). Experimental seizures in rats have confirmed that seizures result into larger motor maps (Henderson et al., 2011; Henry et al., 2008; Ozen et al., 2008; Teskey et al., 2002; Young et al., 2011b; Young et al., 2009; van Rooyen et al., 2006) and lower movement thresholds (Henderson et al., 2011; Young et al., 2009). Laboratory studies have further shown that, seizure induced motor maps expansion are associated with enhanced horizontal fibers synaptic potentiation (Henry et al., 2008; Teskey, 2009) and enhanced overlap of different body parts representations on the sensorimotor cortex as evidenced by multiple movements following electrical stimulation of the sensorimotor cortex (Teskey et al., 2002).

Anti-seizure drugs (ASDs) are the first line drugs in managing seizures and epilepsy. Although the mechanisms of actions of some of the ASDs are not fully known, these drugs control seizures by altering neuronal function in various ways including reducing neuronal excitability and preventing signal transmission. Some studies have shown that some ASDs increase afterdischarge thresholds (ADTs), and lower afterdischarge durations (ADDs) but the effects of these drugs on the movement representations in the motor cortex (motor maps) have not been explored. Since the ASDs control epileptic seizures which are known to change motor map expression, it was speculated that these drugs have effects on motor maps. Therefore, the ASDs by affecting neuronal excitability and synaptic transmissions of the horizontally interconnected networks are likely to directly affect the expression of the movement representations in the neocortex.

Therefore, experiments were designed to explore the effects of ASDs on rat motor maps expression. In vivo experiments using young adult rats were carried out to determine the effects of the ASDs on the forelimb motor maps and movement thresholds (MTs). The experiments
aimed to answer the question of whether acute ASD administration reduces the motor responsive area of the cerebral cortex or not, and to demonstrate the ASDs’ effects on the movement thresholds. These experiments are important because they can give insights into the effect of ASDs on movement representations, neural network function, and as foundational work prior to more research in people with epilepsy.

2.2 Methodology

2.2.1 Subjects

Young adult male Long–Evans hooded rats, weighing about 250 – 450 gm were used in this study. A total of 36 rats were used in this study where 25 rats divided into five groups (each group per drug) were used to test ASDs and 11 rats were used as controls. The rats were housed and handled according to the guidelines set by the Canadian Council for Animal Care. They were given free access to the Lab Diet #5001 (PMI Feeds Inc., St Louis, MO) and water, except for an overnight food fasting prior to an experiment.

2.2.2 Procedures

To derive the motor maps, standard rodent ICMS was performed in rats according to the methodology of Young et al. (2011). All surgeries and ICMS procedures were performed under ketamine and xylazine anesthesia in combination, all of which were given intraperitoneally (i.p.). Initial dosages were ketamine (100 mg/kg) and xylazine (5 mg/kg); and supplemental injections were given as required throughout the procedures either ketamine alone (25 mg/kg), or a cocktail of both ketamine (17 mg/kg) and xylazine (2 mg/kg) so as to maintain a relatively constant level of anesthesia. The anesthesia level was determined by monitoring vibrissae whisking, breathing rate, and foot and tail reflex in response to a gentle pinch.
Anaesthetized rats were secured in a stereotaxic frame with the incisor bar set to skull flat. Longitudinal surgical incision of the scalp was done to expose the skull. Craniotomy was then performed to expose the left sensorimotor neocortex. Windows measuring about 7 mm X 5 mm were made by extending approximately 4 mm anterior to and 3 mm posterior from bregma; and 5 mm lateral of the midline. After craniotomy, the dura was carefully removed. An 18-gauge needle was used to make a small puncture in the cisterna magna before removal of the dura. This was done in order to reduce the intracranial pressure caused by brain edema which likely occurs during craniotomy. The silicone fluid at body temperature was then applied to cover the neocortical surface to keep it from drying. A digital image of the exposed portion of the brain was captured using a Stemi 2000-C stereomicroscope canon digital camera and displayed on a computer where it was then served as canvas image. A grid of 500 µm X 500 µm squares was overlaid on the digital image. ICMSs using tungsten commercial metal electrodes (World Precision Instruments) with a resistance of 1.0 MΩ were performed on the intersections of the grid lines and at a central point in the middle of each square except when located over a blood vessel. This gave an interpenetration distance of 354 µm. The microelectrodes were penetrated at a depth of 1550 µm ± 25 µm. Electrical stimulation was delivered via an isolated stimulator and consisted of 13 biphasic pulses, each 200µs in duration, delivered at a frequency of 333 Hz, and repeated every second.

Attention was made to derive the forelimb motor area. This selection was made because the forelimb movements are easier to observe and more consistent with repeated electrical stimulation than the non-forelimb movements. Forelimb movements include digit, wrist, elbow or shoulder movements whereas non-forelimb movements included trunk, neck, tail, jaw or vibrissae movements. The mapping started at the more central map points and then continued
peripherally to define the forelimb motor area. After initial points in the central area of the map, the points in a horizontal line were derived until a non-responsive point was observed. In a clockwise manner, the forelimb areas were defined. This was done so as to reduce the likelihood of the ICMS affecting the border points of the map. The border of the forelimb motor map was defined by any non-forelimb movement or nonresponsive sites. The nonresponsive point was defined by absence of any movement after a maximum of 60 μA current was delivered. To derive the movement threshold, an electric current was rapidly increased until a movement is detected and then decreased until the movement is no longer present. Rats were maintained in a prone position, with the right forelimb supported by placing an index finger below the forearm and a middle finger behind the elbow for easier visual inspection of all possible forelimb movements. The ICMSs were performed to cover both forelimb areas; namely, the rostral forelimb area and the caudal forelimb area.

To test the effects of various ASDs on the motor maps expression, the drugs were given intraperitoneally (i.p) after the forelimb map had been derived. The ASDs whose effects on motor maps expression were tested were phenytoin (PHT), ethosuximide, valproate, levetiracetam (LEV) and topiramate. PHT (Sigma – Aldrich, Oakville, ON) was given at dosage of 75mg/Kg dissolved in a vehicle consisting of propylene glycol, ethanol and water in a ratio of 4:1:5. The solution made had a concentration of 75mg of PHT per millilitre. Ethosuximide (Sigma – Aldrich, Oakville, ON) dissolved in a saline (300mg/ml) was given at a dose of 300mg/Kg. Valproate (Sigma – Aldrich, Oakville, ON) which was also dissolved in a saline (150mg/ml) was given at a dose of 150mg/Kg. Likewise, LEV (Cayman Chemical Company, Ann Arbor, MI) dissolved in a saline was given at a dose of 250mg/Kg. Topiramate (Cayman Chemical Company, Ann Arbor, MI) dissolved in a DMSO (30mg/ml) was given at a dose of
50mg/Kg. Thirty minutes after i.p. ASD injection, the motor map points were revisited to record new movement types and the MTs. During post-drug mapping, the point where no movement was noted at a 60μA current or higher but initially was responsive at current intensity of 60μA or less was assigned MT of 60 μA. Using similar protocols and amount, the drugs’ vehicles (propylene glycol, ethanol and water in a ratio of 4:1:5, saline and DMSO) were also tested if they have any effect on the MTs and map sizes.

2.2.3 Data analysis

Data recorded during ICMS were entered into excel where mean forelimb map areas and MTs for each rat were calculated. Each responsive site was taken to represent 0.125 mm² of cortical surface (354 × 354 μm), thus the map areas in mm² were calculated by multiplying the number of the responsive points by 0.125 mm². ASDs’or vehicles’ effects on forelimb map sizes and MTs were calculated by subtracting pre-treatment values from post-treatment values. Summarized each rat data was then transported into SPSS version 17 where the means for group of rats and standard error of the mean (SEM) were calculated; and pre- and post-drug map areas and MTs as well as drugs’ effects were compared. The Student paired t-test was used to calculate statistical differences between pre- and post-treatment MTs or map sizes within a group of rats injected with a particular ASD or vehicle. Within group means of changes in map sizes and MTs were compared by one way analysis of variance (ANOVA). Furthermore, ANOVA with Tukey post-hoc tests was used for multiple comparisons testing of means of drugs’ effects on map sizes and MTs. In all cases significance level was set at a two–tailed $p$ value of 0.05. All data are reported as mean ± SEM and summarized in graphs. Asterisks in figures indicate statistically significant effects (* denotes $0.01 < p < 0.05$, ** denotes $p < 0.01$).
2.3 Results

2.3.1 Effect of Phenytoin on Motor Maps Expression

No gross changes in the forelimb motor area of representation in the neocortex was noted post-PHT injection relative to pre-treatment and vehicle controls (figure 2-1A and 2-1B). Quantification of the forelimb maps showed that the changes in forelimb area size in PHT treated rats did not significantly (F(1,9) = 0.006, \( p = 0.94 \)) differ from changes occurred in vehicle control rats (figure 2-1C). PHT slightly reduced the forelimb area from 8.2 ± 0.44 mm\(^2\) to 7.8 ± 0.38 mm\(^2\) (t(4) = 2.372, \( p = 0.08 \)) while the pre- and post-PHT vehicle forelimb area sizes were 6.5 ± 0.75 mm\(^2\) and 6.2 ± 0.08 mm\(^2\) respectively (t(2) = 0.486, \( p = 0.69 \)). Since the non-forelimb borders were not fully explored in deriving the maps, they were not considered in the comparison of the map size.

PHT raised the current required to evoke movements in naïve rats. It significantly (t(4) = -6.114, \( p < 0.01 \)) raised the forelimb MTs as compared to non-significant (t(2) = -2.659, \( p = 0.12 \)) increase caused by the vehicle (figure 2-2A). Increased forelimb MTs post-PHT was noticed in both the distal and proximal forelimb segments. The distal forelimb (digit and wrist) MTs increased from 24.2 ± 1.88 µA to 35.1 ± 1.39 µA (t(4) = -5.691, \( p = 0.01 \)) while the proximal forelimb (elbow and shoulder) MTs increased from 25.6 ± 2.91 µA to 38.2 ± 2.16 µA (t(4) = -2.994, \( p = 0.04 \)). PHT also significantly (t(4) = -5.135, \( p < 0.01 \)), raised the non-forelimb MTs while the vehicle caused no significant (t(2) = -1.025, \( p = 0.41 \)) changes (figure 2-2B).
Figure 2-1: Relatively unchanged forelimb map sizes in response to phenytoin

A Pre-PG:E:W Post-PG:E:W

B Pre-phenytoin Post-phenytoin

Forelimb Points Non-forelimb Points
- Hind limb
- Jaw
- Neck
- Tail
- Whiskers
- Non responsive points

C

Changes in forelimb Areas (mm²)

**Figure 2-1:** Effect of phenytoin on forelimb representation area in the rats neocortex relative to effect caused by its vehicle (propylene glycol, ethanol and water in ratio of 4:1:5) following electrical stimulation at current intensity of 60 µA. Panel A shows pre- and post-vehicle left hemisphere forelimb maps from one of the rats used in the experiments as control. Panel B is pre- and post-phenytoin left hemisphere forelimb maps drawn from a representative rat. Panel C shows quantification of changes in forelimb map sizes (in mm²) post-phenytoin treatment as compared to the changes caused by its vehicle. Both the phenytoin-vehicle and phenytoin caused no gross qualitative changes in forelimb area size in the neocortex (panels A and B respectively). Although there are slight changes in movement types post propylene glycol, ethanol and water solution or post-phenytoin, the overall total forelimb area sizes remained relatively unchanged in both cases (panels A and B). Both the drug and its vehicle caused only a slight decrease in forelimb maps, the changes which were not statistically significant (panel C).
Figure 2-2: Increased movement thresholds caused by phenytoin

**Figure 2-2:** Effects of phenytoin on movement thresholds (MTs) during short train electrical stimulation compared to the effect produced by its vehicle (propylene glycol, ethanol and water in a ratio of 4:1:5). Phenytoin significantly raised the current required to elicit both the forelimb (panel A) and non-forelimb (panel B) movements. Propylene glycol, ethanol and water solution caused no significant changes in movement thresholds.

PG:E:W = propylene glycol, ethanol and water in a ratio of 4:1:5
2.3.2 Effects of Ethosuximide, Valproate and Levetiracetam on Motor Maps Expression

Most forelimb and non-forelimb points in the neocortex turned nonresponsive to electrical stimulation at current intensity of 60 µA following ethosuximide, valproate or LEV injection relative to pre-treatment and post-saline controls (figure 2-3). Ethosuximide, valproate and LEV separately caused significant (F(3,17) =6.688, p < 0.01) reduction in the responsive forelimb area size in the neocortex as compared to changes caused by saline (figure 2-4). While saline caused non-significant (t(2) = -1.896, p = 0.20) changes in forelimb area size, the responsive forelimb area size significantly (t(4) = 5.006, p < 0.01), decreased from 5.83 ± 0.81 mm² to 2.43 ± 0.50 mm² post-ethosuximide injection. Effect of ethosuximide versus effect of saline on forelimb area sizes had a mean difference of -3.59 mm² (p < 0.01). Like ethosuximide, valproate significantly (t (4) = 5.317, p < 0.01) reduced the responsive forelimb area sizes from 6.03 ± 1.11 mm² (pre-treatment) to 3.43 ± 1.42 mm². Post-valproate versus post-saline mean difference in forelimb area changes was -2.81 mm² (p = 0.02). Reduction of forelimb map size post-LEV from 5.60 ± 0.68 mm² to 2.58 ± 0.72 mm² was statistically significant (t(4) = 6.075, p < 0.01), and the effect of LEV as compared to saline effect mean difference was -3.25 mm² (p = 0.01).
Figure 2-3: Representative forelimb maps post-saline, ethosuximide, valproate and LEV

A  Pre-saline  Post-saline

B  Pre-ethosuximide  Post-ethosuximide

C  Pre-valproate  Post-valproate

D  Pre-levetiracetam  Post-levetiracetam

Forelimb Points
- Wrist
- Elbow
- Shoulder
- Digit

Non-forelimb Points
- Hind limb
- Jaw
- Neck
- Tail
- Whiskers
- Non responsive points
**Figure 2-3** demonstrates qualitative changes in forelimb maps from representative rats each following ethosuximide, valproate and levetiracetam treatment relative to pre-treatment and saline controls. Saline was the vehicle for ethosuximide, valproate and levetiracetam. No gross changes in the forelimb motor area of representation in the motor cortex were noted post-saline injection (panel A). Ethosuximide (panel B), valproate (panel C) and levetiracetam (panel D) turned many forelimb and non-forelimb points unresponsive to electrical stimulation at current intensity of 60 µA, leading to smaller forelimb map, the changes which were not noted following saline treatment.
Figure 2-4: Reduction of forelimb areas in response to ethosuximide, valproate and levetiracetam.

Figure 2-4: Variation in forelimb maps size in response to ethosuximide, valproate and levetiracetam as compared to effect caused by their vehicle (saline). Ethosuximide, valproate and levetiracetam each separately caused significant reduction in forelimb maps.
Ethosuximide, valproate and LEV each raised the mean current required to evoke movements in rats. Ethosuximide, valproate and LEV resulted into significantly ($F(3,17) = 17.425, p < 0.01$) higher forelimb MTs compared to changes caused by saline (figure 2-5A). The forelimb MTs remained relatively constant following saline treatment. Pre- and post-saline forelimb MTs were $28.8 \pm 2.45 \mu A$ and $27.9 \pm 1.48 \mu A$ respectively ($t(2) = 0.655, p = 0.58$). Ethosuximide increased forelimb MTs from $29.0 \pm 0.72 \mu A$ to $49.2 \pm 2.02 \mu A$ ($t(4) = -8.308, p < 0.01$) as compared to slightly decreased forelimb MTs caused by saline, making a mean difference of $21.1 \mu A (p < 0.01)$. There was a relatively significant higher increase in the distal forelimb MTs, than in the proximal forelimb MTs following ethosuximide injection. The distal forelimb MTs increased from $29.3 \pm 1.20 \mu A$ to $55.8 \pm 1.59 \mu A$ ($t(4) = -15.056, p < 0.01$) as compared to proximal forelimb MTs which changed from $30.4 \pm 2.01 \mu A$ to $46.4 \pm 2.60 \mu A$ ($t(4) = -4.189, p = 0.01$). Valproate increased the forelimb MTs from $29.1 \pm 1.078 \mu A$ to $54.0 \pm 0.92 \mu A$ ($t(4) = -41.413, p < 0.01$). Raised forelimb MTs post-valproate had a mean difference of $25.7 \mu A (p < 0.01)$ with the slightly decreased forelimb MTs caused by saline. There was no much difference in increased distal forelimb MTs as compared to increase in the proximal forelimb MTs post-valproate treatment. The pre- and post-valproate distal forelimb MTs were $30.0 \pm 0.68 \mu A$ and $57.1 \pm 1.25 \mu A$ respectively ($t(4) = -36.914, p < 0.01$), while the pre- and post-valproate proximal forelimb MTs were $29.3 \pm 1.46 \mu A$ and $53.0 \pm 0.98 \mu A$ ($t(4) = -18.551, p < 0.01$). LEV also increased the current required to evoke forelimb MTs. Forelimb MTs raised from $29.1 \pm 1.00 \mu A$ to $48.7 \pm 2.43 \mu A$ post-LEV treatment ($t(4) = -5.896, p < 0.01$). The mean difference between raised forelimb MTs by LEV ($19.6 \pm 3.33 \mu A$) and the slightly decreased forelimb MTs caused by saline ($-0.9 \pm 1.37 \mu A$) was $20.5 \mu A (p < 0.01$). There was no much difference in raised forelimb MTs post-LEV between the proximal segments of the forelimb and the distal
forelimb segments. The proximal forelimb MTs increased from $28.3 \pm 1.18 \mu A$ to $49.2 \pm 3.02 \mu A$ ($t(4) = -5.274, p < 0.01$) and the distal forelimb MTs raised from $31.1 \pm 1.50 \mu A$ to $48.3 \pm 2.23 \mu A$ ($t(4) = -5.435, p = 0.01$) following LEV injection.

Ethosuximide, valproate and LEV each also significantly ($F(3,17) = 8.528, p < 0.01$) raised the non-forelimb MTs compared to changes caused by saline (figure 2-5B). Non-forelimb MTs non-significantly ($t(2) = -1.876, p = 0.20$) increased from $27.7 \pm 2.27 \mu A$ (pre-treatment) to $32.2 \pm 0.87 \mu A$ post-saline but significantly ($t(4) = -6.233, p < 0.01$) raised from $23.8 \pm 1.93 \mu A$ to $49.9 \pm 2.89 \mu A$ following ethosuximide treatment. The mean difference of increased non-forelimb MTs caused by ethosuximide ($26.1 \pm 4.18 \mu A$) as compared to $4.5 \pm 2.38 \mu A$ increase caused by saline, was $21.6 \mu A$ ($p < 0.01$). Valproate also significantly ($t(4) = -11.670, p < 0.01$) raised the non-forelimb MTs (from $27.9 \pm 1.65 \mu A$ to $55.9 \pm 1.82 \mu A$). The Increased non-forelimb MTs caused by valproate ($28.0 \pm 2.38 \mu A$) was significantly ($p < 0.01$) higher as compared to $4.5 \pm 2.38 \mu A$ increases caused by saline (mean difference = $23.5 \mu A$). LEV raised non-forelimb MTs from $26.3 \pm 1.13 \mu A$ to $54.7 \pm 2.56 \mu A$ ($t(4) = -8.239, p < 0.01$) with the mean change of $28.4 \pm 3.45 \mu A$. The mean change in non-forelimb MTs caused by LEV ($28.4 \pm 3.45 \mu A$) makes the mean difference of $24.0 \mu A$ ($p < 0.01$) with an increase ($4.5 \pm 2.38 \mu A$) caused by saline.
Figure 2-5: Increased movement thresholds caused by ethosuximide, valproate and levetiracetam.
Figure 2-5 shows changes in movement thresholds (MTs) during intracortical microstimulation (ICMS) following pre-treatment with ethosuximide, valproate and levetiracetam relative to changes caused by saline (the vehicle for all three ASDs). Ethosuximide, valproate and levetiracetam each significantly raised the currents required to evoke forelimb movements (panel A). These ASDs also significantly raised non-forelimb MTs (panel B).

** denotes $p < 0.01$. 
2.3.3 Effect of Topiramate on Motor Map Expression

Injection of topiramate into rats resulted into fewer points of forelimb area in the neocortex which responded to electrical stimulation with the current intensity of 60 μA relative to DMSO (figure 2-6A) and pre-treatment (figure 2-6B) controls. Topiramate significantly (t(4) = 3.748, p = 0.02) reduced the responsive forelimb area from 5.10 ± 0.54 mm$^2$ to 3.70 ± 0.60 mm$^2$ while the reduction of forelimb map from 6.88 ± 0.70 mm$^2$ to 6.65 ± 0.87 mm$^2$ post-DMSO was not statistically significant (t(4) = 0.723, p = 0.51). The reduction in the forelimb area size caused by topiramate was significantly (F(1,9) = 5.832, p = 0.04) higher compared to reduction caused by DMSO (figure 2-6C).

Topiramate significantly raised both the forelimb (t(4) = -5.035, p < 0.01) and non-forelimb (t(4) = -9.062, p < 0.01) MTs while DMSO caused non-significant changes in both the forelimb (t(4) = 0.508, p = 0.64) and non-forelimb (t(4) = -0.602, p = 0.58) MTs (figure 2-7). Increased forelimb MTs caused by topiramate (11.5 ± 2.29 μA) was significant (F(1,9) = 18.618, p < 0.01) higher when compared to change caused by DMSO (-0.9 ± 1.73 μA). However, increased non-forelimb MTs (16.5 ± 1.83 μA) did not significantly (F(1,9) = 5.312, p = 0.05) differ from an increase of 3.3 ± 5.45 μA caused by DMSO. Topiramate raised forelimb MTs roughly equally in both the proximal segments of the forelimb and the distal forelimb segments. The proximal forelimb MTs increased from 28.3 ± 1.25 μA to 40.3 ± 2.39 μA (t(4) = -5.865, p < 0.01) and the distal forelimb MTs raised from 29.6 ± 1.97 μA to 45.1 ± 2.75 μA (t(4) = -5.463, p = 0.01) after topiramate treatment.
Figure 2.6: Effects of topiramate on forelimb area in the neocortex

A

Pre-DMSO

Post-DMSO

B

Pre-topiramate

Post-topiramate

Forelimb Points
- Wrist
- Elbow
- Shoulder

Non-forelimb Points
- Hind limb
- Jaw
- Neck
- Tail
- Whiskers
- Non-responsive points

C

Changes in forelimb areas (mm²)

DMSO

Topiramate
**Figure 2-6:** Effect of topiramate on forelimb representation area in the rats’ neocortex following electrical stimulation at current intensity of 60 µA relative to effect of DMSO (topiramate vehicle). Panel A shows qualitative changes in the left hemisphere forelimb map post-DMSO treatment from one of the rats used in the experiments as control. Panel B shows qualitative changes in the left hemisphere forelimb map post-topiramate treatment. In both cases (A and B) maps were re-derived 30 minutes post-DMSO/topiramate. Panel C shows quantification of changes in forelimb map sizes (in mm²) post-topiramate treatment as compared to the changes caused by DMSO. DMSO caused no gross changes in forelimb area size in the neocortex (panels A) while topiramate resulted into fewer forelimb responsive points (panel B). Quantification revealed significant reduction in forelimb area size in the neocortex post-topiramate relative to only a slight reduction caused by DMSO (panel C).
Figure 2.7: Effects of topiramate on forelimb movement thresholds (MTs) and non-forelimb MTs (panels A and B respectively) compared to the effect produced by DMSO, the vehicle in which topiramate was dissolved. Topiramate significantly raised both the forelimb (panel A) and non-forelimb (panel B) MTs while DMSO caused non-significant increase in MTs.
2.4 Discussion

This is the first study to demonstrate that anti-seizure drugs alter motor map expression by reducing forelimb map size as well as increasing movement thresholds in naïve rats. It was found that all ASDs (ethosuximide, valproate, LEV and topiramate) which were tested with the exception of PHT significantly reduced the number of forelimb responsive points in the neocortex relative to appropriate controls. Quantification revealed significant reduction in the forelimb map sizes in response to ethosuximide, valproate, and LEV or topiramate treatment. Furthermore, PHT resulted into smaller forelimb map size although the reduction was not statistically significant. Additionally, these ASDs raised both forelimb and non-forelimb movement thresholds.

Forelimb map size reduction caused by ASDs has a direct relationship with higher post-ASDs MTs which were observed in this study. This reduction may reflect reduced excitability in cortical motor neurons and/or enhanced intracortical inhibition as has previously been reported (Young et al., 2011b). Most of the points which turned unresponsive to electrical stimulation post-ASDs were the ones in the periphery of the maps. These points had relatively higher pre-treatment MTs compared to the points which remained responsive. Thus, ASDs raised their thresholds to elicit movement above 60 µA, making them unresponsive to current intensity of up to 60 µA. Some of these points (data not shown) were unresponsive even at current intensity of 100 µA.

Elevation of thresholds required to elicit forelimb and non-forelimb movements caused by these ASDs supports reports from other studies that ASDs reduce sensorimotor neocortical network output activity, related to movement generation (Borowicz et al., 2003; Gilbert et al., 2003;
For instance, PHT treatment was found to cause significant reduction of muscle stiffness in patients suffering from muscle cramps (Minaker et al., 1989) and it significantly enhanced muscle relaxation in horses (Beech et al., 1988). Other previous studies involving humans and animals have reported increased afterdischarge thresholds (ADTs), and decreased afterdischarge durations (ADDs) following ASDs treatment (Borowicz et al., 2003; Gilbert et al., 2001; Gilbert et al., 2002; Löscher et al., 1998; Lothman et al., 1991). Increased ADTs reflects ASD’s effects on seizure threshold, whereas reduction in ADDs reflects drug’s effects on seizure propagation. Actions of PHT to elevate ADTs were earlier reported by Lothman and colleagues (1991). In other experiments, it was found that acute administration of PHT significantly increased ADTs and reduced ADDs in kindled and non-kindled guinea pigs (Gilbert et al., 2001). Further testing of anticonvulsants’ effects on ADTs and ADDs revealed that phenobarbital and valproate individually significantly raised ADTs and shortened ADDs in the amygdala of kindled guinea pigs, whereas ethosuximide was found to lack effects on both ADTs and ADDs (Gilbert et al., 2002). Study done by Löscher and colleagues (1998) demonstrated shortened ADDs in response to LEV compared to the vehicle controls. Experiments in rats showed that topiramate shortens ADDs and increases ADTs (Borowicz et al., 2003). Further experimental results showed that, co-administration of topiramate with either valproate or phenobarbital resulted in shorter ADDs while co-administration with CBZ significantly shortened ADDs with concomitant increase in ADTs, the effects which were not associated with increased plasma levels of the either drugs (Borowicz et al., 2003).

Alterations of expression of the movement representations in the neocortex by ASDs are likely to be the direct effects of these drugs on neuronal excitability and synaptic transmission.
Therefore, using different mechanisms, ASDs which were tested (PHT, ethosuximide, valproate, LEV and topiramate) could have caused raised MTs by suppressing cortical neuronal activity, leading to either elevating thresholds required to generate action potentials or inhibiting movement signal transmission. The neurons affected may include cortical interneurons and the cortical neurons which project either to the brain stem (corticobulbar) to activate cranial nerves that control head and facial muscles or spinal cord (corticospinal) which activate alpha motor neurons to the body and limb muscles. Evidence of effects of ASDs on corticospinal excitability has previously been reported in human studies (Sohn et al., 2001).

Likewise, ASDs might have caused elevated MTs by inhibiting depolarization of cranial nerves that control facial muscles and spinal cord alpha motor neurons that control body and limb muscles or impairing transmission at neuromuscular junction. ASDs have been reported to inhibit signal transmission at neuromuscular junction. For example, in one study ethosuximide and carbamazepine were found to cause reduction of both miniature end-plate potential (MEPP) and end-plate potential (EPP) while PB reduced only MEPP amplitude with no statistically significant effect on EPP amplitude (Alderdice and Trommer, 1980). Elevation of threshold for movement elicitation by some ASDs such as ethosuximide which affects calcium currents could be a result of reduced skeletal muscle excitability. Apart from studied effect of ethosuximide on smooth muscle activity there is limited literature regarding direct effects (if any) of other ASDs on muscle cells. Ethosuximide causes smooth muscle cell hyperpolarization and thus muscle relaxation. Experiments in rats have proved that ethosuximide induce smooth muscle hyperpolarization by activating Ca2+-dependent –K+ channels leading to K+ efflux (Kristev et al., 1994; Velkova et al., 1995). Hyperpolarization effect of ethosuximide explains this drugs’
effect to induce vasodilatation (Velkova et al., 1995). This drug might also cause skeletal muscles hyperpolarization leading to difficulty in initiation of movement.

PHT is thought to have raised MTs by stabilizing the inactivated state of voltage-gated sodium channels (LaRoche and Helmers, 2004; Macdonald, 1989; Schwarz and Grigat, 1989; Wakamori et al., 1989; Yaari et al., 1986), thus making them difficult to generate movement signals (action potentials), although the drug has been found to lack effect on the amplitude and duration of individual action potentials (McLean and Macdonald, 1986b). PHT inhibitory effects may also be due to enhancing GABA-mediated inhibition (Granger et al., 1995; Macdonald and McLean, 1982) as well as reducing both neuronal calcium uptake (Macdonald and McLean, 1982; Messing et al., 1985) and excitatory synaptic transmission (Griffith and Taylor, 1988). Elevation of MTs post-ethosuximide could be caused by its inhibitory activity on low-threshold T-type calcium currents in thalamic neurons (Coulter et al., 1989a; Coulter et al., 1989b; Macdonald and Kelly, 1995; Meldrum, 1996).

Valproate could have raised MTs by a number of mechanisms. These mechanisms include reduction of the neuronal low-threshold T-type calcium currents (Kelly et al., 1990), blocking voltage-sensitive sodium channels (Macdonald and Kelly, 1993; Macdonald and Kelly, 1995; McLean and Macdonald, 1986b) and facilitation of GABA inhibitory effects as scholars have shown that, valproate causes significant increase in the GABA content of the brain by increasing GABA synthesis (Johannessen, 2000; LöScher, 1999) and inhibition of nerve terminal GABA-inactivation (Johannessen, 2000). Other studies have suggested that the acute depressant effect of valproate is most likely due to a potentiation of postsynaptic GABA receptor action rather than increasing the brain GABA level (Farrant and Webster, 1989). Reduction in neuronal
activity post valproate could also be caused by its effect on weakening neuronal excitation induced by NMDA-type glutamate receptors (LöScher, 1999).

Although the mechanisms of action of LEV are poorly understood, this drug raised MTs probably by reducing release of excitatory neurotransmitters through alteration of vesicle fusion (Yang et al., 2007) or/and enhancement of GABA mediated inhibition (Doelken et al., 2010).

Like other ASDs, topiramate also caused significant raise in both forelimb and non-forelimb MTs, the cause that I can relate with its inhibitory effect on voltage-dependent sodium channels (DeLorenzo et al., 2000; McLean et al., 2000) or potentiation of GABAergic receptors (White, 1997) or antagonizing NMDA–glutamate receptors (Meldrum, 1996; Shank et al., 1994).

The effects of ASDs to reduce the forelimb map sizes and increase MTs are in agreement with previous researches that have established an inverse relationship between movement thresholds and motor map size (Brown et al., 2011; Henderson et al., 2011; Scullion et al., 2013; Young et al., 2009). While seizures result into larger motor maps (Henry et al., 2008; Lado et al., 2002b; Lee et al., 2009; Ozen et al., 2008; Teskey et al., 2002; Uematsu et al., 1992; Urasaki et al., 1994; van Rooyen et al., 2006; Young et al., 2009; Henderson et al., 2011) and lower movement thresholds (Henderson et al., 2011; Young et al., 2009), smaller maps are associated with increased movement thresholds (Brown et al., 2011; Scullion et al., 2013; Young et al., 2011b). Motor maps expansion can be caused by enhanced horizontal fibers synaptic potentiation (Henry et al., 2008; Teskey, 2009) or due a loss of GABAergic inhibition (Jacobs and Donoghue, 1991). Therefore, ASDs by inhibiting release of excitatory neurotransmitters or enhancing GABAergic inhibition in the cortical horizontal fibres lead into smaller maps which are associated with increased thresholds for movement geneartion.
In conclusion, the present study has shown that ASDs raise the thresholds required to elicit movements and cause map hypotrophy in naïve rats, the effects which are caused by inhibitory effects of these drugs on the cortical motor neurons and probably the alpha motor neurons to the muscles or direct effects on muscle activity. The observed anticonvulsants’ effects on motor maps expression are unlikely due to anaesthetic effects because anaesthetic level monitoring protocols of drug experiments were similar to those of control groups. The study was conducted using naïve rats because the findings may be extrapolated to predict how the use of ASDs in patients with epilepsy might alter motor maps expression in the health regions of the brains. The effects of ASDs using an animal model of epilepsy, is also important information but I did not carry such long-term experiments. However, currently there is a student in Dr. Teskey’s laboratory who is examining how ASDs (I tested in naïve rats) affect motor map expression in kindled rats.
CHAPTER THREE

ACUTE EFFECTS OF BUMETANIDE ON KINDLED SEIZURES, MOTOR MAP EXPRESSION AND LAYER V PYRAMIDAL CELLS ACTIVITIES OF YOUNG ADULT RATS

Authors:

Haruna I. Dika¹,², Ahmed Hussin¹, Jeffrey A. Boychuk ³, Omid Javizian¹, Quentin J. Pittman¹,4, G. Campbell Teskey¹,4,5*

¹Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta, Canada, T2N 4N1
²Department of Physiology, Catholic University of Health and Allied Sciences, Mwanza, Tanzania
³Department of Physiology, University of Kentucky College of Medicine
⁴Department of Physiology and Pharmacology, University of Calgary, Calgary, Alberta, Canada, T2N 4N1
⁵Department of Cell Biology and Anatomy, University of Calgary, Calgary, Alberta, Canada, T2N 4N1

Haruna I. Dika – Conducted ICMS experiments in naïve rats, analyzed data and prepared the manuscript

Ahmed Hussin and Jeffrey A. Boychuk - Conducted slice electrophysiological studies and analyzed their data

Omid Javizian – Conducted experiments in kindled rats and his analyzed data
Abstract

Purpose: To determine the potential anti-seizure activity of bumetanide and its effect on cellular and network cortical excitability in post-neonatal and young adult rats.

Methods: Using a standard kindling paradigm young adult male Long–Evans hooded rats were kindled in the corpus callosum once per day for 15 days. On kindling days 2, 5 and 15 rats were intraperitoneally injected with bumetanide or vehicle (saline) and 30 minutes later their afterdischarge thresholds (ADTs), afterdischarge durations (ADDs) and seizure stages were determined. Post-bumetanide ADTs, ADDs and seizure stages were then compared to pre-treatment and post-saline controls. Intracortical microstimulation (ICMS) was also performed in naïve and 3Hz-kindled rats where the motor maps were derived by recording movement types and their thresholds. This was then followed by intraperitoneal injection of bumetanide or saline and re-deriving the maps after 30 minutes. Post-bumetanide or saline movement thresholds were compared to the baseline values. Finally, layer V pyramidal cell slices were prepared from young (P22-30) naïve rats for whole cell electrophysiological recordings. The electrophysiological recordings (resting membrane potential, action potential (AP) threshold and number of APs) were done before and 30 minutes after bath application of bumetanide. Pre- and post-bumetanide electrophysiological parameters were then compared.

Results: Bumetanide increased ADTs and reduced seizure severity compared to saline controls effects. Movement thresholds were significantly increased in both naïve and 3Hz-kindled bumetanide-treated rats with no significant changes in the forelimb map sizes. In the slice, bumetanide increased the minimum current required to elicit action potentials and hyperpolarized the cells. It also reduced the rate of firing of the layer V pyramidal cells.
Significance: The findings demonstrate that, acute administration of bumetanide has significant anti-seizure activity. The present study also provides the first evidence that bumetanide can reduce synaptic and network motor cortical excitability measures which may be related to movement generation. These pre-clinical findings provide support for bumetanide’s efficacy as an anticonvulsant in people with epilepsy.

Key words: Movement thresholds, afterdischarge duration, seizure stage
3.1 Introduction

Animal and human studies have suggested that diuretics may be of value in the management of seizures and epilepsy (Maa et al., 2011). One of the diuretics, bumetanide, has shown anti-seizure effects in neonates (Kahle et al., 2009; Mareš, 2009; Mazarati et al., 2009) but its anti-seizure mechanisms are not fully understood. Despite of unclear anti-seizure mechanisms of bumetanide this drug when used in combination with classical ASDs increases effectiveness of seizure control by more than 100 % (Dzhala et al., 2008). Anti-seizure effects of this drug are thought to be through its inhibitory action on the two cations chloride transporter, the Na⁺-K⁺-2Cl⁻ cotransporter (NKCC1) which is highly expressed in young neurons and decreases with age (Payne et al., 2003; Wang et al., 2002; Dzhala et al., 2005). NKCC1 is an electroneutral transporter which accumulates chloride ions in immature neurons. The neurons with high intracellular chloride ions depolarize in response to activation of GABA_A receptors due to efflux of negatively charged chloride ions (Payne et al., 2003). This is different from adult neurons which hyperpolarize in response to GABA_A receptor activation. Adult neurons have lower intracellular chloride ions due to diminished NKCC1 and increased expression of K⁺-Cl⁻ cotranspoter (KCC2) which is the chloride exporter. Therefore activation of GABA_A receptors in adult neurons leads to chloride influx and thus hyperpolarization (Kaila, 1994). Blockage of NKCC1 by bumetanide therefore lowers intracellular Cl⁻ ion concentrations thus reversing the GABA depolarizing effect on immature brain neurons as well as in other neurons which accumulate Cl⁻ ions within the cell (Blumenfeld, 2002; Misgeld et al., 1986).

However, increased expression of NKCC1 may occur in insulted adult neurons (Huberfeld et al., 2007; Munoz et al., 2007; Reid et al., 2013), which suggests that these cells
may also be targets of bumetanide. Additionally, bumetanide is implicated in interfering activation of NMDA receptors (Beck et al., 2003) and on glutamate release where it inhibits vesicular uptake of glutamate (Roseth et al., 1995). These additional effects of bumetanide suggest that this drug may also have anti-seizure effects even in mature brains; and recently it has been reported that long term use of bumetanide reduces frequency of seizures in adult patients with temporal lobe epilepsy (Eftekhar et al., 2013). Other indicators that bumetanide may inhibit matured neurons and thus potential anticonvulsant activity in adults is the fact that it has shown anxiolytic effect in adult rats (Krystal et al., 2012). Apart from inhibiting neuronal functioning, bumetanide has neuroprotective role where it prevents neuronal death (Jantzie et al., 2015; Shulga et al., 2012). Bumetanide prevents myelin basic protein loss and neuronal degeneration (Jantzie et al., 2015). In addition, bumetanide also prevents upregulation of pan-neurotrophin receptor p75(NTR). Increased p75(NTR) and its interaction with other factors makes injured neurons to depend on brain-derived neurotrophic factor (BDNF) for survival. Thus, bumetanide dissociates neural survival from dependency on BDNF (Shulga et al., 2012).

As pointed earlier in chapters one and two, seizures alter motor map expression leading to lower movement thresholds and larger motor maps. Thus, the drugs which control seizures are likely to restore the altered motor maps or change un-altered maps. Although bumetanide has shown promising role in managing seizure activity by affecting neuronal function, its mechanisms of actions are not fully known and study of its effect on motor maps expression has been sparse. There is limited information about the effect of bumetanide on the movement thresholds and the functioning of the motor cortex in general. Since bumetanide has shown promising role in managing seizure activity by affecting neuronal function, it was speculated that
this drug, like the classic anti-seizures would affect expression of the motor maps in the neocortex. Furthermore, there is limited information which indicate that bumetanide reduces seizure frequency in adult patients and its role in controlling seizures in adults has not been given attention. In general, anti-seizure mechanisms of bumetanide are poorly understood and whether it has anti-seizure activity in adults is largely unknown. Additionally, the effects of bumetanide on neuronal networks (motor maps) are completely unknown. Therefore, this study was designed to show whether bumetanide could be an effective anti-seizure drug in adults with neocortical foci or not. The experiments were set to examine the effects of bumetanide on afterdischarge thresholds (ADTs), afterdischarge durations (ADDs) and seizure behavior in young adult rats kindled using the ‘standard’ kindling technique. Additionally, this study explored the effects of bumetanide on motor maps using young adult naïve and 3Hz-kindled rats. The study provides the first evidence of how bumetanide affects the neuronal networks that control movements. The experiments using rats aimed to indirectly show whether bumetanide reverses the changes in motor maps, which occur in patients with epilepsy. Motor maps changes may be utilized as biomarkers in monitoring progression of epileptogenesi and motor disturbances. Furthermore, the effects of bumetanide on the young rats’ layer V pyramidal cells membrane potentials, action potential thresholds and firing rates were tested. This experiment was designed to give insights into the effect of bumetanide on movement representations, neural network function, and as foundational work prior to more research in people with epilepsy.

Since seizures alter motor map expression and bumetanide controls seizures by affecting neuronal function, it was speculated that bumetanide would directly affect the neuronal networks. Due to the interconnected networks of neurons, particularly the local horizontal connections of layer V pyramidal neurons that make up the corticospinal tracts, it was
hypothesized that: 1. bumetanide would raise ADTs and reduce both the ADDs and severity of
seizures, 2. it would directly alter motor maps expression by raising movement thresholds and
reducing map sizes, and 3. it would inhibit activity of layer V pyramidal neurons.

3.2 Materials and Methods

3.2.1 Animals

A total of 39 post-neonatal and young adult male Long–Evans (LE) hooded rats (Charles
River; Montreal, QC) were used in this study. Ten bumetanide treated and six control (saline
treated) young adult rats weighing about 250 – 450 gm were used for standard kindling
experiments to assess ADTs, ADDs and seizure severity. For testing effect of bumetanide on
MTs and forelimb map area, four naïve and seven 3Hz-kindled young adult rats (about 250 – 450
gm) were used; and three naïve young adult rats were used as controls. Nine young (P22-30)
naïve rats were used for electrophysiological studies. All rats were housed in clear plastic cages
in a colony room that was maintained on a 12 hours on/12 hours off light cycle. All experiments
were conducted during the light phase. The rats were housed and handled according to the
guidelines set by the Canadian Council for Animal Care. They were given free access to the Lab
Diet #5001 (PMI Feeds Inc., St Louis, MO) and water, except for an overnight food fasting prior
to an experiment.

3.2.2 Implantation of Electrodes for Kindling

Using Teflon-coated, stainless steel wires, twisted wire bipolar electrodes were
constructed to a diameter of 178 μm (A-M Systems, Everett, WA, USA). The ends of the wire
were stripped of the Teflon coating and connected to a gold plated male amphenol pin. 0.5 mm
separated between each pole of the electrodes. Prior to the surgical procedure the rats were
anaesthetized with 5% isoflurane and maintained at 2% isoflurane for the remainder of the
procedure. If the rats began gasping, the isoflurane was reduced to 1% until gasping had disappeared after which it was raised back to 2%. Following the administration of 5% isoflurane, rats were secured in a stereotaxic frame with the incisor bar set to skull flat. A subcutaneous injection of lidocaine (2%) was then injected at the incision site. Longitudinal surgical incision of the scalp was done on the dorsum of the head and the skin gently pushed to the periphery to expose the skull surface.

The electrodes were implanted in the hemisphere ipsilateral to the rat’s dominant forelimb according to the rat stereotaxic coordinates (Swanson, 1992). The stimulating electrode was implanted into the corpus callosum (1.0 mm anterior to the line of bregma, 0.5 mm lateral to the midline and 3.5-4.5 mm ventral from the brain surface) and the recording electrode was implanted in the frontal neocortex, which corresponds to the caudal forelimb area. The poles of the recording electrode were separated such that one pole was in layer V and the other in layer III of the neocortex (1.0 mm anterior to bregma, 4.0 mm lateral to the midline and 1.5 mm ventral from the brain surface). Using electrophysiological monitoring during electrode implantation, the depth of the electrode was adjusted to maximize the amplitude of the evoked response. The amphenol pins were then inserted into a nine-pin McIntyre connector plug and the electrodes were cemented and anchored to the skull with five stainless steel screws and dental cement. The rats were given 7 days of rest following the surgery before continuing with experimentation.

3.2.3 Standard Kindling

Prior to any kindling, on the first day of kindling, the ADT was determined. The ADT was defined as the weakest current that was required to elicit an afterdischarge ≥ 7 s. Beginning at 25 µA, the current was increased by 25 µA increments with 120 s intervals until the ADT was determined. Kindling stimulation which consisted of a 1s train of 60 Hz biphasic rectangular
pulses, 1 ms in duration and separated by 1 ms, was delivered at an intensity of 100 μA above that required to elicit an ADT. Kindling sessions occurred once a day, until 15 sessions were completed. Electroencephalographs (EEGs) were recorded from the recording electrode in the frontal neocortex and the stimulating electrode in the corpus callosum. Following each kindling session, a five stage behavioral scale was used to assess seizure stages. Seizure stages were scored as such: (0) freezing accompanied by electrographic seizure; (1) motor arrest accompanied by facial automatisms such as vibrissae twitching; (2) head nodding and chewing; (3) unilateral forelimb clonus; (4) rearing on hindlimbs and bilateral forelimb clonus; (5) rearing, clonus of all four limbs, and falling.

3.2.4 Testing Bumetanide Effects on ADTs, ADDs and Seizure Severity

On days 2, 5, and 15, bumetanide or saline were injected intraperitoneally (i.p). Bumetanide (Sigma – Aldrich, St. Louis, MO, USA) dissolved in a saline was given at a dose of 2.5 mg/Kg (Mareš, 2009; Mazarati et al., 2009). The solution made had a concentration of 2.5 mg of bumetanide per millilitre. Drug solutions were prepared and used on the daily basis. The control group rats were given 2.5 ml of saline per Kg body weight. The ADTs, ADDs and seizure stages were then determined 30 minutes post bumetanide or saline injection. Post-bumetanide and post-saline means ADTs, ADDs and seizure stages were then compared.

3.2.5 3Hz- Kindling

Kindling stimulation which consisted of a 120 s train of 3Hz biphasic rectangular pulses, 1 milliseconds (ms) in duration and separated by 1 ms, was delivered at an intensity of 1000 μA. Using electroencephalography, EEGs were recorded from the recording electrode in the frontal neocortex and the stimulating electrode in the corpus callosum. 3Hz-kindling sessions occurred twice a day, with each session being at least 4 hours apart for a total of 2 consecutive days.
Therefore, there were a total of 4 kindling sessions per rat. Following the 4th session, rats underwent the intracortical microstimulation (ICMS) protocol and mapping protocol after being given the night to rest.

### 3.2.6 Craniotomy and Intracortical Microstimulation

Craniotomy and ICMSs were performed following the same protocols and procedures as described in chapter two above.

### 3.2.7 Testing Bumetanide Effects on MTs and Forelimb Map Areas

To test the effects of bumetanide on the motor maps expression, the drug or vehicle (saline) were given intraperitoneally after the forelimb map had been derived. Bumetanide (Sigma – Aldrich, St. Louis, MO, USA) dissolved in a saline was given at a dose of 2.5 mg/Kg (Mareš, 2009; Mazarati et al., 2009). The solution made had a concentration of 2.5 mg of bumetanide per millilitre. Drug solutions were prepared and used on the daily basis. The control group rats were given 2.5 ml of saline per Kg body weight. Thirty minutes after i.p. bumetanide or saline injection, the motor map points were revisited to record new movement types and the thresholds in which they were evoked.

### 3.2.8 Slice Preparation

Naïve male LE rats (P22-30) were anaesthetized using isoflurane and then decapitated. The brain was quickly removed and placed (for several minutes) in ice-cold slicing solution containing (in mM) 87 NaCl, 2.5 KCl, 25 NaHCO₃, 0.5 CaCl₂, 7 MgCl₂, 1.25 NaH₂PO₄, 25 glucose, and 75 sucrose saturated with 95% O₂/5% CO₂. The brain was then blocked and mounted on a vibrating slicer (Leica, Nussloch, Germany) and submerged in ice-cold slicing solution. Coronal slices (300 µm) containing motor cortex were cut from a region extending about 900 µm anterior and 300 posterior to bregma. Slices were then incubated at 32°C for 30
min in artificial cerebral spinal fluid (aCSF) containing (in mM) 126 NaCl, 2.5 KCl, 26 NaHCO₃, 2.5 CaCl₂, 1.5 MgCl₂, 1.25 NaH₂PO₄, 10 glucose; saturated with 95% O₂/5% CO₂. Following incubation, slices were maintained in aCSF at room temperature (21° C – 24° C) for at least 30 min before recording.

3.2.9 Electrophysiological Recordings

Slices were transferred to a recording chamber and perfused with 32° C – 34° C aCSF at a flow rate of 1–2 ml/min. Whole-cell recordings were obtained from Layer V pyramidal cells visualized with an AxioskopII FS Plus (Zeiss; Oberkochen, Germany) upright microscope with a X40 objective using infrared differential interference contrast optics. The layer V pyramidal cells were identified based on their tear-drop/triangular morphology with large apical dendrites orientated to the pial surface as well as electrophysiological characteristics (Bandrowski et al., 2003; Brill and Huguenard, 2010). Whole-cell recordings were obtained using borosilicate glass microelectrodes (tip resistance 3–5 MΩ) filled with a solution containing (in mM) 108 K-gluconate, 8 Na-gluconate, 2 MgCl₂, 8 KCL, 1 potassium EGTA, 4 potassium ATP, 0.3 sodium GTP and 10 HEPES that was corrected to pH 7.2 with KOH. Recordings were accepted for analysis if series resistance was <20 MΩ and changes in access resistance were <25% throughout the experiment. Electrophysiological signals were amplified using the Multiclamp 700A amplifier (Molecular Devices, Union City, CA, USA), low-pass filtered at 1 kHz, and digitized at 10 kHz using a Digidata 1322A (Molecular Devices). Data were collected and stored on a computer for offline analysis using Clampfit 9 (Molecular Devices).

Slice-ICMS was conducted as previously described by Scullion et al. (2013). Current clamp recordings were used to assess the pyramidal cell’s responses during stimulation of the
slice with parameters that are typical of the ICMS used to assess movement representations in anaesthetized rodents (Brown et al., 2011; Flynn et al., 2010; Young et al., 2011a; Young et al., 2011b). Slices were stimulated by an extracellular electrode (0.50 MΩ microelectrode tungsten TM33A05; World Precision Instruments Inc.) positioned directly dorsal (about 130 µm) to each layer V pyramidal cell prior to recording from the latter in whole-cell configuration. Electrical stimulation was delivered to the slice via an isolated stimulator (A-M Systems; Carlsborg, WA, USA) and consisted of 13 monophasic cathodal pulses, each 200 µs in duration, delivered at a frequency of 333 Hz, and repeated every second. Slice-ICMS current intensity was manually increased from 0 µA to 60 µA in 5 µA increments each given for 10 seconds, i.e., each increment administered for 10 trains with each 1Hz train consisting of 13 pulses.

Pyramidal cell responses to slice-ICMS were assessed with the following measures; (1) The minimum intensity of slice-ICMS current needed to evoke an action potential (AP), referred to as initial AP Threshold, (2) The number of APs at each slice-ICMS current intensity, assessed as the mean number of APs during the 10 ICMS trains given per intensity, (3) The resting membrane potential (RMP) which was measured several minutes following whole-cell configuration and immediately prior to ICMS stimulation of the slice. Furthermore, we measured the number of APs fired to an intracellular current step (50 nA; 4 s).

3.2.10 Testing Bumetanide Effects on Layer V pyramidal Cells

All electrophysiological recordings were done before and after bath application of bumetanide (10 µM; Sigma-Aldrich, St. Louis, MO, USA). Post-bumetanide cell recordings were performed 30 minutes following the start of application. Stock solutions of both drugs were stored at -20°C until use.
3.2.11 Statistical Analysis

In the standard kindling experiments, mean ADTs, ADDs and seizure stages on days 2, 5, and 15 were determined after saline or bumetanide treatment. A Wilcoxon Signed Ranks Test was performed to make comparisons between differences in ADTs, ADDs and seizure stage between saline and bumetanide treated rats. Data recorded during ICMSs was entered into Microsoft excel where forelimb and non-forelimb mean MTs for each rat were calculated. Summarized each rat data was then transported into SPSS where the means for group of rats and standard error of the mean (SEM) were calculated; and pre- and post-bumetanide or saline MTs were compared. The Student paired t-test was used to calculate statistical differences. Forelimb responsive points were used to draw the forelimb maps. For electrophysiological studies, cell recordings were averaged across neurons (i.e. n = number of neuron) for each condition. Significance of difference was determined using a Student’s T test or ANOVA with post hoc comparisons based on what was appropriate for the number of conditions and/or measurements. Wilcoxon Signed Rank Test was used to assess differences in the number of APs elicited at each current intensity of slice-ICMS. In all experiments data are reported as Mean ± SEM and significance level was set at a two-tailed p value of 0.05. Data are also summarized in graphs.

3.3 Results

3.3.1 Effects of Bumetanide on ADTs, ADDs and Seizure Severity

There were no significant changes in ADTs, ADDs or seizure stage on days 2 and 5 of standard kindling in both bumetanide treated rats and saline control rats (figure 3-1). On day 15 of kindling, pre-treatment with bumetanide resulted into significantly (t (8,5)= 2.42, p = 0.04) higher ADTs (255.6 ± 47.1 µA) as compared to 137.5 ± 12.5 µA of saline treated rats (figure 3-
1A) with no significant \((t(8, 5) = 1.82, p = 0.10)\) difference in the ADDs changes between the two groups (figure 3-1B). On day 15 of kindling, there was also significant \((t(8,5) = 2.36, p = 0.04)\) reduction in the severity of seizure following pre-treatment with bumetanide (to stage 3.3 ± 0.4) as compared to stage 4.5 ± 0.2 which was observed in saline control rats group (figure 3-1C).

3.3.2 Effects of Bumetanide on Motor Maps Expression in the neocortex

3.3.2.1 Effects of Bumetanide on Forelimb Representations

Acute administration of bumetanide like saline in which it was dissolved, caused no gross changes in the forelimb area sizes in both naïve and 3Hz-kindled rat groups (figure 3-2). Quantification of the forelimb maps showed that the forelimb map size did not significantly change post-bumetanide injection both in the naïve rats \((t(3) = 1.225, p = 0.31)\) and in the kindled rats group \((t(6) = 1.375, p = 0.22)\). Pre- and post-bumetanide forelimb maps in naïve rats were 6.4 ± 0.82 mm\(^2\) and 5.2 ± 0.67 mm\(^2\) respectively and in kindled rats were 7.2 ± 0.35 mm\(^2\) and 6.6 ± 0.33 mm\(^2\) respectively. Forelimb map sizes in naïve rats also remained relatively unchanged \((t(2) = -1.896, p = 0.20)\) following saline treatment. Pre- and post-saline forelimb map sizes were 4.9 ± 0.33 mm\(^2\) and 5.1 ± 0.40 mm\(^2\) respectively.
Figure 3-1: Increased ADT and reduced seizure severity by bumetanide with no significant change in ADD
**Figure 3-1:** Changes in after-discharge thresholds (ADTs) [panel A], after-discharge durations (ADDs) [panel B] and severity of seizure [panel C] in bumetanide pre-treated rats relative to saline treated control rats. There were no statistically significant differences in ADTs, ADDs and seizure stages on days 2 and 5 of standard kindling in bumetanide treated versus control rats. On day 15 there was a statistically significant increase in ADTs [panel A] and reduction in seizure stage [panel C] in the bumetanide treated rats group versus saline control group with no significant difference in reduction of ADDs caused by bumetanide and that caused by saline [panel B]. ADTs, ADDs and seizure stages are given as Mean± standard error of the mean [SEM].

* denotes p < 0.05, Error bars represent SEM
3.3.2.2 Effects of Bumetanide on MTs

Bumetanide raised the mean current required to evoke movements in naïve and 3Hz-kindled rats. Forelimb MTs significantly increased in both naïve rats (t(3) = -4.391, p = 0.02) and in 3Hz-kindled rats group (t (6) = -3.035, p = 0.02) in response to bumetanide treatment while forelimb MTs in naïve rats remained relatively unchanged (t(2) = 0.655, p = 0.58) post-saline treatment (figure 3-3A). Bumetanide increased forelimb MTs by 56.2 % in naïve rats and 28.0 % in kindled rats as compared to a slightly decreased forelimb MT of 3.2 % caused by saline, making differences of about 59.4% and 31.2 % respectively.

Bumetanide also caused significant increase in the non-forelimb MTs in naïve rats (t(3) = -4.128, p = 0.03) and in 3Hz-kindled rats (t(6) = -2.694, p = 0.04) while saline caused no significant change (t(2) = -1.876, p = 0.20) in non-forelimb MTs (figure 3-3B). The percentage differences of increased non-forelimb MTs caused by bumetanide in naïve rats (114.4 %) and in 3Hz-kindled rats (41.9 %) as compared to 16.2 % increase caused by saline, were about 98.2 % and 25.7 % respectively.
Figure 3-2: Lack of gross changes in forelimb map in response to bumetanide treatment

Figure 3-2: Representative maps showing effect of bumetanide on forelimb representation in the rats’ neocortex relative to saline control effect. (A) Pre- and post-saline left neocortical forelimb maps drawn during ICMS of one of the control rats. (B) Pre- and post-bumetanide left neocortical forelimb maps of a representative non-kindled rat. (C) Pre- and post-bumetanide left neocortical forelimb maps of a representative 3Hz-kindled rat. The post-saline/bumetanide maps were re-derived 30 minutes post-treatment. The points were considered non-responsive, if no movement was observed at current intensity of 60 µA. Both bumetanide and saline caused no
gross changes in the forelimb motor map size despite of some changes in movement type post-bumetanide/saline.
Figure 3-3: Increased movement thresholds by bumetanide

A

Forelimb MTs (μA)

Saline  Bu-NK  Bu-3HK

B

non-forelimb MTs (μA)

Saline  Bu-NK  Bu-3HK

■ Pre-treatment  □ Post-treatment
Figure 3-3 shows changes in forelimb (panel A) and non-forelimb (panel B) movement thresholds (MTs) in naïve and 3Hz kindled rats following bumetanide treatment compared to post-saline changes MTs. Saline is the vehicle in which bumetanide was dissolved. MTs are given as Mean ± standard error of the mean (SEM). Post-bumetanide forelimb and non-forelimb MTs were significantly higher as compared to pre-treatment values in non-kindled (naïve) and 3Hz-kindled rats, while saline did not cause significant changes in both forelimb and non-forelimb MTs. Bu-NK = bumetanide treated non-kindled rats group, Bu-3HK = bumetanide treated 3Hz-kindled rats group.

* denotes p < 0.05, Error bars represent SEM.
3.3.3  Effects of Bumetanide on Layer V pyramidal cells

Bumetanide application (10 µM) altered the responses of layer V pyramidal cells within slices of motor cortex to ICMS stimulation (figure 3-4). The minimum ICMS current required to trigger firing of layer V pyramidal neurons was increased after bath application of bumetanide relative to pre-treatment required current (figure 3-4A). Measurement of single neurons AP threshold showed that, bumetanide significantly (t(9) = 3.617; p < 0.01) raised the threshold for AP generation (figure 3-4B). The pyramidal neurons membranes became relatively more hyperpolarized post-bumetanide (figure 3-4C), although the difference between pre-treatment and post-bumetanide RMP was not statistically significant (t(9) = 1.7; p = 0.13). The number of cells in the slice which fired in response to electrical stimulation were relatively less after bath application of bumetanide than to pre-treatment number (figure 3-4D). Cells exhibited a significantly (Wilcoxon Signed Rank; 1-tail; Z-value=-2.981; p < 0.01) lower number of APs following application of bumetanide (Figure 3-4E). Further experiments showed that, application of bumetanide reduces the number of APs fired to an intracellular current injection (figure 3-5). The number of APs fired to an intracellular current step (50 nA; 4 s) was significantly (t(11) = 5.340; p < 0.001) reduced by bumetanide (figure 3-5B).
Figure 3-4: Inhibition of cortical layer V pyramidal cells by bumetanide
**Figure 3-4** Demonstrates effect of bumetanide on response of layer V pyramidal cells of motor cortex to electrical stimulation. Panel A shows two representative current clamp traces showing the responses of a layer V pyramidal cell before (left) and after (right) bath application of bumetanide (10µM). The black arrow denotes the first action potential elicited by ICMS stimulation (initial AP threshold). The inset located at the top left region of the trace shows expanded view of the initial action potentials elicited by slice-ICMS. Panel B illustrates pooled data of the ICMS stimulation AP thresholds that elicit the first AP for each cell. Bumetanide significantly increased the thresholds for generation of AP. Panel C shows the pooled data of resting membrane potential measured two minutes after whole-cell configuration and 30 minutes after bumetanide application. Bumetanide made the resting membrane potentials of the neurons more negative, although the changes were not statistically significant. Panel D summarizes the group data of the proportion of cells firing at each 5 µA increment of ICMS stimulation and panel E summarizes the group data of the total number of action potentials evoked at each 5 µA increment of ICMS stimulation for each cell. Bumetanide resulted into few cells firing in response to ICMS current (panel D) and fewer APs generated by these neurons (panel E) at each current intensity. The five panels in this figure (A-E) all demonstrates that application of the bumetanide decreases firing of layer V pyramidal cells of motor cortex.

* denotes p < 0.05, Error bars represent SEM.
Figure 3-5: Reduced number of APs fired to an intracellular current injection post-bumetanide treatment.

Figure 3-5: Response of Layer V pyramidal neurons to an intracellular current step (50nA; 4s). Panel A shows two representative current clamp traces showing the responses of a layer V pyramidal cell to the current injection before (left) and after (right) bath application of bumetanide (10μM) and panel B shows pooled data of the number of APs fired post-bumetanide relative to pre-treatment. Bumetanide caused significant reduction in the number of APs fired. ** denotes p < 0.01, Error bars represent SEM.
3.4 Discussion

The present study has shown that bumetanide raises ADTs, and reduces severity of seizures with no significant changes of ADDs in young adult rats. Bumetanide also significantly increased both the forelimb and non-forelimb MTs in naïve and 3Hz kindled young adult rats with no significant effect on forelimb map size. It also inhibited the young rats’ cortical layer V pyramidal neurons by hyperpolarizing them, raising threshold for AP generation and reducing their firing rate. These findings show that, bumetanide has significant anti-seizure activity and supports previous studies which showed that, bumetatine suppresses seizure activity (Dzhala et al., 2008; Eftekhari et al., 2013; Kahle et al., 2009; Mareš, 2009; Mazarati et al., 2009).

The increased ADTs and reduction of seizure severity following bumetanide treatment supports findings which were reported in a newborn case study (Kahle et al., 2009) and in experiments done in neonatal rats (Mareš, 2009; Mazarati et al., 2009). Kahle and colleagues (2009) noted significant reductions in mean seizure duration and frequency in six weeks old baby following treatment with bumetanide. Mareš (2009) found bumetanide to suppress tonic phase of generalized seizures and shorten latencies of seizures in 12-day-old rats. Mazarati and colleagues (2009) experiments in neonatal rats showed bumetanide effect on increasing ADTs and shortening ADDs during electric stimulation of the hippocampus. In addition to increased ADTs and reduced ADDs, bumetanide delayed the occurrence and reduced the number of full motor seizures during kindling (Mazarati et al., 2009). In the current study, we observed a significant increase in ADTs and reduction in seizure severity on day 15 of kindling, but not on days 2 and 5. This could be due to increased NKCC1 expression which is likely to occur as a result of kindling. NKCC1 which is highly expressed in immature neurons, tend to decrease with
age, and in rats it normally start declining after postnatal day 14 and almost completely disappear after postnatal day 21 (Payne et al., 2003; Wang et al., 2002). However, its expression increases in insulted mature neurons, in which kindling is one of the causes (Huberfeld et al., 2007; Munoz et al., 2007; Reid et al., 2013; Okabe et al., 2002). Therefore in adult neurons of the kindled rats, increased NKCC1 which are target of bumetanide will cause significant inhibition to these neurons. Although NKCC1 levels were not determined in this study, its expression was expected to be higher on kindling day 15, than on days 2 and 5, and thus significant inhibitory effects of bumetanide.

Lack of significant reduction of ADDs observed in our investigation which is in contrary to findings by Mazarati and colleagues (2009) could be due to the differences on seizure origin which depended on brain regions where actual stimulations were done. Mazarati and colleagues stimulated the hippocampus while in our study stimulation was done in the corpus callosum. This may be explained by the differential distribution of the NKCC1 transporters in the brain. NKCC1 is the target for inhibitory effect of bumetanide. The hippocampus is rich in NKCC1 expression while pyramidal neurons in layer V of the neocortex do not show a detectable amount of NKCC1 (Kanaka et al., 2001).

The effects of bumetanide are closely related to the effects ASDs on the excitability of neuronal network (Borowicz et al., 2003; Gilbert et al., 2001; Gilbert et al., 2002; Löscher et al., 1998; Lothman et al., 1991) where it has been found that ASDs raise ADTs and lower ADDs. For instance phenytoin significantly raises ADTs (Gilbert et al., 2001; Lothman et al., 1991) and reduces ADDs (Gilbert et al., 2001). Phenobarbital and valproate also cause increased ADTs and shortened ADDs in the amygdala of kindled guinea pigs (Gilbert et al., 2002). Increased ADTs and decreased ADDs by topiramate have also been demonstrated (Borowicz et al., 2003).
The present results provide the first evidence that bumetanide can alter sensorimotor neocortical network output activity, which may be related to movement generation, by increasing both forelimb and non-forelimb MTs in naïve and 3Hz-kindled rats. Decreased in the neuronal excitability observed in this study is in agreement with the results from previous studies (Bos et al., 2011; Kahle et al., 2009; Mareš, 2009; Mazarati et al., 2009). These effects are likely due to inhibition of layer V pyramidal cells by bumetanide as it was observed in electrophysiological recordings in this study.

Although the increased MTs post-bumetanide observed in naïve rats are likely due to the inhibitory effect of bumetanide on the NKCC1 co-transporter, reversing the excitatory effect of GABA on the brain neurons, there might be other unknown mechanisms. We speculate the existence of additional mechanisms because the excitatory effects of GABA in the rat brain were reported to diminish after two weeks post-natal, due to upregulation of KCC2 and downregulation of NKCC1 (Dzhala et al., 2005; Wang et al., 2002). While the mechanisms responsible for increased MTs post-bumetanide in naïve rats are unclear, in 3Hz-kindled rats the raise in MTs could mainly be due inhibitory effect of this drug on NKCC1 as there is a likely increase of NKCC1 expression following kindling. This is because experiments have shown that seizures reduce KCC2/NKCC1 ratio (Huberfeld et al., 2007; Munoz et al., 2007). These changes can result in intracellular chloride accumulation and reappearance of excitatory effect of GABA like in the immature neurons. Therefore inhibiting NKCC1 would cause a rise in MTs by inhibiting GABA excitatory effects.

Bumetamide also inhibits vesicular uptake of glutamate (Roseth et al., 1995) and activation of NMDA receptors (Beck et al., 2003). Inhibiting vesicular uptake of glutamate leads to impaired glutamate release at excitatory synapses. This interference with excitatory synaptic
transmission might also have contributed to raising intensity of currents required to evoke movements in both naïve and kindled adult rats. Increased movement thresholds could be a result of hyperpolarization effect of bumetanide. Bumetanide hyperpolarizes the cells by increasing cell membranes permeability to potassium by activating calcium dependent potassium channels (Wang et al., 2013). Bumetanide, through unknown mechanisms triggers a rise in intracellular calcium ions which leads to activation of calcium dependent potassium channels (Wang et al., 2013). Additionally, increased movement thresholds in both naïve and kindled rats post-bumetanide could be a result of hypokalemia induced by diuretic action of bumetanide. Bumetanide inhibits NKCC2 transporters which are highly and almost exclusively expressed in the renal tubular epithelial cells (Wittner et al., 1991). Inhibition of NKCC2 causes diuresis by increasing potassium, sodium and chloride loss in urine; the effect which can lead to decreased extracellular potassium concentration (Halstenson and Matzke, 1983). A low extracellular potassium level promotes potassium efflux leading to hyperpolarization of cells. Hyperpolarization reduces neuronal and skeletal muscle excitability and thus increasing thresholds for current required to elicit movement.

Since bumetanide has shown anticonvulsant activities by increasing MTs and ADTs, it was expected to cause smaller map sizes as previous studies which have demonstrated inverse relationship between the map size and movements thresholds (Brown et al., 2011; Henderson et al., 2011; Scullion et al., 2013; Young et al., 2011b; Young et al., 2009). Increased movements thresholds are associated with smaller maps (Brown et al., 2011; Scullion et al., 2013; Young et al., 2011b) while larger maps are associated with lowered movement thresholds (Henderson et al., 2011; Young et al., 2009). However, the current study has not provided evidence of reduction in forelimb responsive area (maps) in the neocortex despite of elevated movement
thresholds. Lack of significant alteration in the forelimb map size by bumetanide suggests that bumetanide has no significant effect on synaptic transmission in the cortical horizontal fibers or chronic bumetanide administration might be required to induce long term synaptic depression, leading to reduction in map size. Motor maps size are affected by horizontal fibers synaptic potentiation (Henry et al., 2008; Teskey, 2009). Therefore, enhanced horizontal fibers synaptic potentiation leads to motor maps expansion while interfering with horizontal fibres synaptic transmission through inhibiting release of excitatory neurotransmitters cause map hypotrophy. Lack of effect of bumetanide in ADDs (which is the marker of signal propagation in brain) observed in the current study also supports that bumetanide is ineffective in preventing horizontal signal transmission and thus lack of effect on motor maps size.

We observed hyperpolarization of cortical layer V pyramidal neurons, increased threshold for AP generation and reduce firing rate of these neurons post-bumetanide treatment. Although there is limited literature about effects of bumetanide specifically on the layer V pyramidal cells’ resting membrane potential, AP threshold and firing rate, these findings are in line with the results from a study done by Bos and colleagues (2011) who found decreased neural discharge after bumetanide treatment.

We can conclude that, this study has demonstrated that bumetanide has significant anticonvulsant activities despite of its mechanisms being unclear. Increased ADTs post-bumetanide reflects its effects on seizure threshold whereas lack of significant reduction in ADDs reflects that bumetanide is less effective in preventing seizure propagation. Further experiments need to be done in human to demonstrate the efficacy of bumetanide as anticonvulsant.
3.5 Acknowledgements

This work was funded by and NSERC grant to G.C.T. and CIHR grants to Q.J.P. and G.C.T.

Disclosure of Conflicts of Interests: None of the authors has any conflict of interest to disclose.

We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.
CHAPTER FOUR

EFFECTS OF ANTI-SEIZURE DRUGS ON SEIZURE INDUCED SEVERE HYPOXIC EPISODE (SISHE)

4.1 Introduction

Seizures cause alteration in brain function (Blennow et al., 1977; Friedman and Dingledine, 2011; Howse et al., 1974; Ingvar and Siesjo, 1983; Meldrum and Nilsson, 1976) and structures (Holmes, 2002; Holmes et al., 2002), the changes which are the likely causes of various neurological symptoms which occur post-seizures. Recently, it has been discovered that seizures induce severe brain hypoxia (pO\textsubscript{2}<10mmHg) (Teskey and Farrell, 2012), the effect which might also play a causative role of neurological symptoms occurring post-seizure. Teskey and Farrell (2012) found that experimental seizure-induced severe hypoxic episode (SISHE) occurs only in those areas of the brain participating in the seizure. Development of SISHE is associated with a localized reduction in cerebral blood flow. SISHE not only occurs in experimental animals but also in people with epilepsy (Dr. Paolo Federico, unpublished data). This phenomenon can be prevented by pretreatment by two classes of vasodilator agents; calcium channel blockers such as nifedipine, and cyclo-oxygenesase-2 enzyme inhibitors such as acetaminophen. While nifedipine is effective in preventing SISHE when given before and after seizures, acetaminophen is only effective when given before seizures (Wolff, Farrell and Teskey, unpublished). The duration of this phenomenon (30-100 minutes) depends on seizure duration (Teskey and Farrell, 2012), with longer seizures related to more severe hypoxia and of longer duration. Thus, SISHE is likely to be caused by a cascade of events which lead vasoconstriction.
Epileptic seizures are controlled by anti-seizure drugs (ASDs) which work by preventing neuronal action potential generation and their transmission. Therefore, ASDs by affecting neuronal excitability and synaptic transmission may alter the functioning of neurovascular unit which is thought to be involved in the regulation of cerebral blood flow. This neurovascular unit consists of neurons, glia and microvessels (Iadecola, 2004). Alteration of neurovascular functioning will thus alter cerebral perfusion and oxygen supply. In addition to this, studies have shown that some ASDs promote recovery from ischemic strokes (Crumrine et al., 1997; Smith and Meldrum, 1995; Wiard et al., 1995; Graham et al., 1993) while others delay the recovery or worsen the strokes (Brailowsky et al., 1986; Goldstein, 1995; Goldstein et al., 1990; Hernandez and Russell, 1992). For instance, immediate administration of lamotrigine (LTG) after ischemic stroke reduces the size of the formed cortical infarct and improves neurological deficits (Smith and Meldrum, 1995) and the drug is being used in combination with other drugs to manage patients with ischemic stroke (Chen et al., 2002). On the other hand, phenytoin (PHT) and phenobarbital (PB) have been found to delay recovery of motor functions after stroke (Brailowsky et al., 1986; Goldstein, 1995; Goldstein et al., 1990; Hernandez and Russell, 1992). Furthermore, PB has been shown to reduce cerebral blood flow (Goddard-Finegold and Armstrong, 1987; Goddard-Finegold et al., 1990), the effect which may worsen ischemic stroke although it is protective in hemorrhagic stroke. Because of these varying effects of ASDs on ischemic stroke, it was speculated that these drugs may affect SISHE differently; others preventing it while others worsen it.

Despite of the above mentioned effects of ASDs on cerebral blood flow and ischemic stroke, there is no documented information on whether ASDs prevent development or reduce severity of SISHE or not. Therefore, the aim of the present study was to investigate the possible effects of
anti-seizures on SISHE. *In vivo* experiments using young adult rats were carried out to determine the effects of the ASDs and a putative ASD, bumetanide, on hippocampal seizure-induced hypoxic episodes. The hippocampus was chosen because it is often the site of seizure initiation in temporal lobe epilepsy, which is the most common form of adult focal epilepsy. Findings from this study could provide potentially important information for clinicians.

4.2 Methods

4.2.1 Subjects

Young adult male Long–Evans hooded rats (N = 9) were used in this study. At least five rats were used to test each drug’s effects. The rats were obtained from Charles River; Montreal, QC. All rats were housed in clear plastic cages in a colony room that was maintained on a 12 hours on/12 hours off light cycle. All experiments were conducted during the light phase. The rats were housed and handled according to the guidelines set by the Canadian Council for Animal Care. They were given free access to the Lab Diet #5001 (PMI Feeds Inc., St Louis, MO) and water.

4.2.2 Implantation of Electrodes and Oxygen Probes

Rats were implanted with a bipolar stimulating/recording electrode and oxygen-sensing device (optrode). Stimulating/recording bipolar electrodes measuring 178 μm in diameter were constructed as described in chapter three above. The electrode and optrode were surgically implanted under stereotaxic control and anchored to the skull following similar procedures and protocols as described in chapter three above.

The stimulating and recording electrodes as well as oxygen-sensing device were all permanently implanted in the right dorsal hippocampus (CA1) of each animal according to the
stereotaxic coordinates of Swanson (1992). The electrode was implanted in the dorsal hippocampus (3.3 mm posterior to bregma, 2.0 mm lateral to midline and 3.8 mm deep from the skull surface). Thus, seizure induction and electroencephalography (EEG) recording were done in the dorsal hippocampus. The optrode was implanted in the right dorsal hippocampus (4.0 mm posterior to bregma, 4.0 mm lateral to midline and 4.0 mm deep from the skull surface) and adhered to the skull with dental cement. After surgical implantation of electrodes and oxygen probes, the rats were allowed a week recovery before the next phase of experimentation which involved kindling and measuring of hippocampal oxygen levels.

4.2.3 Seizure Inductions and Oxygen Recordings

Electrical stimulations were done daily according to the methodology of van Rooyen et al. (2006). After connecting the electrodes and optrode to the rat about 5 minutes time was given for the rat to settle down and hippocampal pO$_2$ to stabilize. Current intensity of 500 µA was delivered through stimulating electrode which was implanted into the right dorsal hippocampus. The stimulation consisted of 1 second train of 60 Hz biphasic rectangular pulses, 1ms duration and separated by 1 ms. An EEG was recorded before, during and post-electrical stimulation so as to establish the seizure durations. About 10 seconds EEG recordings were done before electrical stimulation to establish the baseline EEG activity. Depending on pre-set EEG recording speed, durations of seizure activity in seconds were determined. Hippocampal oxygen levels were also recorded before, during and for 90 to 120 minutes after the stimulation. After stabilization of hippocampal pO$_2$, a minimum of 100 seconds O$_2$ recordings were done before electrical stimulation to establish the baseline pO$_2$ level. Post-ictal hippocampal oxygen recording period depended on time taken for baseline O$_2$ recovery. For many rats, this took about 90 minutes while for few rats it took up to 120 minutes.
Hippocampal oxygen levels were measured using the implanted fiber-optic oxygen-sensing device (optrode). This optrode uses blue light pulses to induce fluorescence of a platinum luminophor that is quenched by oxygen at the silicon/rubber tip within a local area (~500 µm³). The technology (Oxylite, Oxford, UK) is superior to other pre-existing technologies as it does not consume oxygen while measuring it. Each biologically inert probe, called an optrode, is individually calibrated by the manufacturer and its accuracy was verified before use. In our laboratory the accuracy was tested in two ways, measuring oxygen level in oxygen depleted saline and measuring pO₂ in a dying rat. In the first method, saline was put in a beaker and the optrode inserted into the beaker to measure pO₂ in saline. Oxygen was then depleted by introduction of Nitrogen air bubbles in saline. After depletion of oxygen by Nitrogen, pO₂ as measured by optrode decreased to almost zero mmHg. In the second method, hippocampal pO₂ was measured by the optrode in fully awake rate and the rat was then euthanized by high dose of pentobarbital, in which hippocampal pO₂ was observed to drop to zero mmHg. Once positioned, the probe provides accurate and continuous measurements of local pO₂ levels in brain tissue in fully awake freely moving animals over several months. The recorded oxygen profile was served in the computer which was connected to the optrode machine.

4.2.4 Testing the effects of ASDs on SISHE

Testing the effects of ASDs on seizure-induced hypoxia was done on rats that developed SISHE. Rats who failed to develop severe hypoxia in the hippocampus (pO₂ <10 mmHg) after one week of 60 Hz electrical stimulation were excluded from study. The drugs were given intraperitoneally 30 minutes before electrical stimulation. The ASDs whose effects on SISHE development were tested were phenytoin (PHT), phenobarbital (PB), ethosuximide, valproate,
levetiracetam (LEV), topiramate, lamotrigine (LTG) and the putative ASD, bumetanide. PHT (Sigma – Aldrich, Oakville, ON) was given at dosage of 75mg/Kg dissolved in a vehicle consisting of propylene glycol, ethanol and water in a ratio of 4:1:5. The solution made had a concentration of 75mg of PHT per millilitre. PB (University of Calgary, HSC-ARC) dissolved in saline (30mg/ml) was given at a dose of 50 mg/Kg. Ethosuximide (Sigma – Aldrich, Oakville, ON) dissolved in saline (300mg/ml) was given at a dose of 300mg/Kg. Valproate (Sigma – Aldrich, Oakville, ON) which was also dissolved in saline (150mg/ml) was given at a dose of 150mg/Kg while LEV (Cayman Chemical Company, Ann Arbor, MI) dissolved in saline was given at a dose of 250mg/Kg. Topiramate (Cayman Chemical Company, Ann Arbor, MI) dissolved in DMSO (30mg/ml) was given at a dose of 50mg/Kg while LTG (Cayman Chemical Company, Ann Arbor, MI) dissolved in DMSO (15mg/ml) was given at a dose of 15 mg/Kg and bumetanide (Sigma – Aldrich, Oakville, ON) which was also dissolved in DMSO (2.5mg/ml) was given at a dose of 2.5 mg/Kg. In addition to ASDs, nifedipine, the calcium channel blocker, was also tested as a positive control.

Some rats were used for testing more than one drug. Rats that were used to test more than one ASD were given 2 – 3 days (depending on half-life of the previously used drug) of free drug before testing another drug. Re-using of the rats depended on their baseline hippocampal oxygen levels and re-recorded post-seizure with no-drug treatment hippocampal oxygen profile. Prerequisite for re-use of the rat was noticing of no significant change in baseline oxygen level and no-drug seizure induced hypoxic level. Therefore, only the rats, whose baseline oxygen levels and no-drug seizure induced hypoxic level remained relatively unchanged, were re-used for testing other ASDs.
4.2.5 Statistical Analysis

Statistical analyses were conducted using Graph Pad Prism 5 (Graph Pad Software Inc., La Jolla, CA). Hippocampal oxygen profiles recorded using the optrode and served on the computer were transformed into text files. The text files were entered into Graph Pad Prism 5 where the oxygen profiles (mmHg against time in minutes) were drawn for each rat. Areas below 10 mmHg for each rat were then calculated. Hippocampal oxygen profiles for the groups of rats (average) were plotted using Graph Pad Prism. The means of kindle (pre-treatment), post-vehicle and post-ASD of areas below 10 mmHg, primary seizure duration, and baseline hippocampal pO$_2$ were each determined and separately analyzed by repeated measures analysis of variance (ANOVA). When significant ANOVAs were obtained post-hoc analysis was performed using Tukey's Multiple Comparison Testing. Statistical significance levels were fixed at two-tailed $p$-value of 0.05. Data are presented as mean ± SEM. Asterisks in figures represent significance level: * $p < 0.05$ and ** $p < 0.01$.

4.3 Results

4.3.1 Effects of Nifedipine on SISHE and Seizure Duration

Pretreatment with the calcium channel blocker nifedipine altered the pO$_2$ profile relative to vehicle and kindled control rats without significantly (F(2,14) = 1.4, $p = 0.30$ ) altering seizure duration (Figure 4-1A). Nifedipine also resulted in a significant (F(2,14) = 37.62, $p < 0.01$) increase in baseline pO$_2$ levels (Figure 4-1B) as well as significantly (F(2,14) = 17.05, $p = 0.01$) less severe hypoxia (Figure 4-1C) relative to vehicle and kindled controls.
Figure 4-1: Prevention of SISHE by nifedipine
**Figure 4-1:** Changes in hippocampal pO$_2$ with time in response to seizures after nifedipine or vehicle treatment as compared to post-seizure pO$_2$ in kindle (no-drug treatment). Seizures were induced 30 minutes post-nifedipine or vehicle treatment and pO$_2$ recording was started 100 s before seizure inductions. Graphs in panel A show changes in pO$_2$ over time. Each of the trace (kindle/pre-treatment, post-vehicle and post-nifedipine) represents average pO$_2$ (n = 5) at a particular point of time. The averages of pO$_2$ recorded for each rat before seizure induction (in each condition) were used to calculate the means (n = 5) baseline pO$_2$ which are potted in panel B. Severity of hypoxia post-seizure was quantified by calculating area below 10 mmHg (pO$_2$ in mmHg X time in minutes ) for each rat’s trace. The calculated areas were used to determine the means (n = 5) and potted in panel C. Panel (A) shows that nifedipine prevents development of severe hypoxia (pO$_2$ < 10 mmHg) without significant changes in seizure duration. Nifedipine pre-treatment caused a significant increase in the baseline hippocampal oxygen levels as shown by the recordings of oxygen partial pressures in the right hippocampus of rats (panel B). Panel (C) shows that, nifedipine pre-treatment resulted in a significant reduction in the severity of seizure induced hypoxia.

** denotes $p < 0.01$
4.3.2 Effects of Phenytoin on SISHE and Seizure Duration

PHT did not alter the hippocampal post-seizure pO$_2$ profile relative to vehicle and kindled control rats and did not cause a significant (F(2,14) = 2.10, $p = 0.19$) change in seizure duration (Figure 4-2A). PHT also did not cause significant (F(2,14) = 3.51, $p = 0.08$) change in the hippocampal baseline pO$_2$ levels (Figure 4-2B). Additionally, it lacked significant (F(2,14) = 3.43, $p = 0.08$) effect on reduction of seizure induced hypoxia severity (Figure 4-2C) relative to vehicle and kindled controls.

4.3.3 Effects of Phenytoin on SISHE and Seizure Durations

PB did not alter the hippocampal post-seizure pO$_2$ profile relative to vehicle and kindled control rats. Post-PB the mean seizure duration was relatively longer (F(2,14) = 5.28, $p = 0.04$) compared to pre-treatment and post-vehicle controls (figure 4-3A) but Tukey’s post-hoc multiple comparison showed lack of significant ($p > 0.05$) differences in seizure durations. Pre-treatment with PB did not significantly change both the hippocampal baseline pO$_2$ levels (F(2,14) = 26.43, $p = 0.93$) and severity of hypoxia (F(2,14) = 0.10, $p = 0.90$) as shown in figure 4-3 (panels B and C respectively).

4.3.4 Effects of Ethosuximide on SISHE and Seizure Duration

Pretreatment with ethosuximide raised the hippocampal post-seizure pO$_2$ profile relative to vehicle and kindled control rats without significantly (F(2,17) = 1.22, $p = 0.34$) altering seizure duration (Figure 4-4A). Ethosuximide also resulted in a significant (F(2,17) = 9.8, $p < 0.01$) increase in the hippocampal baseline pO$_2$ levels (Figure 4-4B) as well as significant (F(2,17) = 8.72, $p < 0.01$) reduction in the severity of seizure induced hypoxia (Figure 4-4C) relative to vehicle and kindled controls.
Figure 4-2: Seizure induced hypoxia and seizure durations post-phenytoin treatment

A

B

C

Seizure Duration (s)

0 20 40 60 80

Kindle

Vehicle

Phenytoin

Hippocampal pO₂ (mmHg)

0 10 20 30 40

Time (min)

0 20 40 60 80 100

Baseline Hippocampal pO₂ (mmHg)

0 10 20 30 40

Kindle Vehicle Phenytoin

Severity of Hypoxia (mmHg*min)

0 100 200 300 400 500 600

Kindle Vehicle Phenytoin
Figure 4-2 demonstrates changes in hippocampal pO$_2$ and seizure durations post-phenytoin treatment. Graphs in panel A show changes in pO$_2$ over time. Each of the trace (kindle/pre-treatment, post-vehicle and post-phenytoin) represents average pO$_2$ (n = 5) at a particular point of time. The averages of pO$_2$ recorded for each rat before seizure induction (in each condition) were used to calculate the means (n = 5) baseline pO$_2$ which are potted in panel B. Severity of hypoxia post-seizure was quantified by calculating area below 10 mmHg (pO$_2$ in mmHg X time in minutes) for each rat’s trace. The calculated areas were used to determine the means (n = 5) and potted in panel C. This figure shows that seizures caused a hypoxic period that was not significantly modulated by phenytoin (panel A). Panel A further shows that seizure durations did not significantly change in response to phenytoin treatment. Phenytoin did not cause a significant change in the baseline hippocampal oxygen levels (panel B). Phenytoin and its vehicle caused only a slight reduction in seizure induced hypoxia (panel C), the reductions which were not statistically significant.
Figure 4-3: Unchanged SISHE severity in response to phenobarbital
Figure 4-3 shows seizure induced hypoxia and seizure durations in response to phenobarbital. Graphs in panel A show changes in pO$_2$ over time. Each of the trace (kindle/pre-treatment, post-vehicle and post-phenobarbital) represents average pO$_2$ (n = 5) at a particular point of time. The averages of pO$_2$ recorded for each rat before seizure induction (in each condition) were used to calculate the means (n = 5) baseline pO$_2$ which are potted in panel B. Severity of hypoxia post-seizure was quantified by calculating area below 10 mmHg (pO$_2$ in mmHg X time in minutes) for each rat’s trace. The calculated areas were used to determine the means (n = 5) and potted in panel C. Phenobarbital did not alter the pattern of seizure induced hypoxia in rats (panel A). Post-phenobarbital seizure duration was relatively longer compared to both pre-treatment and post-vehicle values (panel A) but post-hoc multiple comparisons testing showed lack of statistically significance differences in seizure durations. No significant changes in the baseline (before seizure induction) hippocampal oxygen levels were observed 30 minutes post-phenobarbital treatment (panel B). Likewise, the severity of seizure induced hypoxia was not significantly altered by phenobarbital as shown by lack of significant changes in the size of area below 10 mmHg in hippocampal pO$_2$ profile recorded over time (panel C).
Figure 4-4: Reduction in SISHE severity caused by ethosuximide
Figure 4-4: shows post-seizure right hippocampal pO$_2$ trend over time following ethosuximide injection as compared to post-vehicle and kindle (no-drug treatment) profiles. Each of the trace (kindle/pre-treatment, post-vehicle and post-ethosuximide) represents average pO$_2$ (n = 6) at a particular point of time. The averages of pO$_2$ recorded for each rat before seizure induction (in each condition) were used to calculate the means (n = 6) baseline pO$_2$ which are potted in panel B. Severity of hypoxia post-seizure was quantified by calculating area below 10 mmHg (pO$_2$ in mmHg X time in minutes) for each rat’s trace. The calculated areas were used to determine the means (n = 6) and potted in panel C. Panel A shows that post-ethosuximide profile is slightly higher than pre-treatment and post-vehicle profiles with no significant differences in seizure durations between post-ethosuximide and post-vehicle and pre-treatment controls. Panel B shows significant increase in the baseline (pre-seizure) hippocampal pO$_2$ 30 minutes after intraperitoneal injections of ethosuximide. Finally, Panel C shows significant reduction in mean area below 10 mmHg in response to ethosuximide treatment. This signifies reduction in severity of seizure induced hypoxia. ** denotes $p < 0.01$
4.3.5 Effects of Valproate on SISHE and Seizure Duration

Post-seizure hippocampal pO$_2$ profile in valproate pre-treatment rats did not change relative to those of vehicle and kindled control rats and the drug did not cause significant (F(2,14) = 0.6596, p = 0.54) changes in seizure duration (Figure 4-5A). No significant (F(2,14) = 1.256, p = 0.33) changes in hippocampal baseline pO$_2$ levels were noted in response to valproate injections (figure 4-5B) and the severity of seizure induced hypoxia was not significantly (F(2,14) = 0.9637, p = 0.42) altered by this drug (Figure 4-5C) when compared to vehicle and kindled controls.

4.3.6 Effects of Levetiracetam on SISHE and Seizure Duration

LEV did not alter pre- and post-seizure hippocampal pO$_2$ profile relative to vehicle and kindled control rats and it caused non-significant (F(2,14) = 1.204, p = 0.35) change in seizure duration (Figure 4-6A). Unaltered hippocampal pO$_2$ profile by LEV, is depicted by lack of significant changes in baseline pO$_2$ levels (F(2,14) = 0.9717, p = 0.42) and severity of seizure induced hypoxia (F(2,14) = 0.2537, p = 0.78) relative to vehicle and kindled controls as shown in figure 4-6B and figure 4-6C respectively.
Figure 4-5: Effect of valproate on SISHE severity and seizure durations

A

Seizure Duration (s)

0  20  40  60  80

Kindle

Vehicle

Valproate

Hippocampal pO2 (mmHg)

Time (min)

B

Baseline Hippocampal pO2 (mmHg)

Kindle  Vehicle  Valproate

C

Severity of Hypoxia (mmHg/min)

Kindle  Vehicle  Valproate
**Figure 4-5:** Panel A shows relatively unchanged trends of hippocampal pO₂ after seizure and a non-significant change in the durations of seizures post-valproate treatment compared to its vehicle and kindle (pre-treatment) levels. Panel B demonstrates a non-significant alteration in the hippocampal pO₂ 30 minutes post-valproate injections. Panel C shows a tendency to worsen the severity of seizure induced hypoxia post-valproate treatment as quantified by the area below 10 mmHg in the hippocampal pO₂ profile, but the changes were not statistically significant.
Figure 4-6: Unchanged SISHE severity in response to levetiracetam
Figure 4-6: Panel A demonstrates seizure induced hypoxia and seizure durations in response to LEV and its vehicle. LEV caused no gross changes in pO$_2$ profile relative to prê-treatment and post-vehicle profiles and it caused non-significant changes in seizure duration. LEV also caused non-significant changes in hippocampal baseline pO$_2$ (panel B) and severity of seizure induced hypoxia (panel C).
4.3.7 Effects of Topiramate on SISHE and Seizure Duration

Topiramate did not alter the hippocampal post-seizure pO\textsubscript{2} profile relative to vehicle and kindled control rats and it caused non-significant (F(2,14) = 0.4221, \( p = 0.67 \)) change in seizure duration (Figure 4-7A). Topiramate significantly (F(2,14) = 16.54, \( p < 0.01 \)) increased the hippocampal baseline (pre-seizure) pO\textsubscript{2} (figure 4-7) but it’s effect on baseline pO\textsubscript{2} was almost similar to its vehicle’s effect. Despite of observed increase in baseline pO\textsubscript{2} following topiramate injection, this drug did not significantly (F(2,14) = 2.343, \( p = 0.16 \)) reduce the severity of seizure induced hypoxia (Figure 4-7C) relative to vehicle and kindled controls.

4.3.8 Effects of Lamotrigine on SISHE and Seizure Durations

Pretreatment with LTG did not cause significant alteration of post-seizure hippocampal oxygen profile when compared to pre-treatment and vehicle controls (figure 4-8A). LTG also did not cause significant (F(2,14) = 2.353, \( p = 0.16 \)) change in seizure duration (Figure 4-8A). It also caused non significant changes in the hippocampal baseline pO\textsubscript{2} level (F(2,14) = 0.021, \( p = 0.98 \)) [Figure 4-8B] as well as in severity of seizure induced hypoxia (F(2,14) = 1.336, \( p = 0.32 \)) relative to vehicle and kindled controls rats (figure 4-8C).

4.3.9 Effects of Bumetanide on SISHE and Seizure Duration

Like the classic anti-seizure drugs, the putative anticonvulsant, bumetanide neither prevented nor worsened the seizure induced hypoxia and the seizure durations (figure 4-9). Bumetanide did not altered the post-seizure pO\textsubscript{2} profile relative to vehicle and kindled control rats and it caused no significant (F(2,14) = 0.4717, \( p = 0.64 \)) change in seizure duration (Figure 4-9A).
Figure 4-7: Non-significant change in SISHE severity and seizure duration post-topiramate
**Figure 4-7:** Seizure induced hypoxia and seizure durations in response to topiramate and its vehicle (panel A). Panel B demonstrates significant increase in the baseline hippocampal oxygen partial pressure in response to topiramate and its vehicle. However, the differences between topiramate and vehicle effects were not significant. Panel C shows non-significant changes in severity of seizure induced hypoxia following topiramate and vehicle treatment.

** denotes \( p < 0.01 \), ns = no significant difference
Figure 4-8: Seizure induced hypoxia and seizure durations post-lamotrigine

A

Seizure Duration (s)
0 20 40 60 80
Kindle
Vehicle
Lamotrigine

Hippocampal pO2 (mmHg)

Time (min)

B

Baseline Hippocampal pO2 (mmHg)

Kindle  Vehicle  Lamotrigine

C

Severity of Hypoxia (mmHg/min)

Kindle  Vehicle  Lamotrigine
Figure 4-8: Lamotrigine caused no gross changes on hippocampal pO$_2$ profile with no statistically significant change in seizure duration (panel A). Baseline (no seizure) hippocampal oxygen levels remained relatively unchanged 30 minutes post-lamotrigine (panel B). Lack of significant alteration in hippocampal pO$_2$ by lamotrigine (panel A) is depicted as non-significant change is the severity of SISHE quantified by size of area below 10 mmHg (panel C).
Figure 4-9: Lack of changes in SISHE severity and seizure durations post-bumetanide.
**Figure 4-9**: Seizure induced hypoxia and seizure durations in response to bumetanide and its vehicle (DMSO). Bumetanide did not alter the hippocampal pO₂ profile and seizure duration (panel A). Panel B shows relatively unchanged baseline (no seizure) hippocampal oxygen levels in rats 30 minutes post-bumetanide treatment. Panel C demonstrates a non-significant reduction in seizure induced severe hypoxia following bumetanide treatment.
Bumetanide also resulted in non-significant changes in both the hippocampal baseline pO$_2$ levels (F(2,14) = 1.145, $p = 0.37$) and severity of seizure induced hypoxia (F(2,14) = 0.899, $p = 0.44$) relative to vehicle and kindled controls, as shown in figure 4-9B and figure 4-9C respectively.

4.3.10 Summary of the Study Findings

Nifedipine, the calcium channel blocker, did not cause significant alteration in the seizure durations but significantly increased the hippocampal baseline pO$_2$ and effectively prevented development of severe hypoxia after seizure (figure 4-1). Ethosuximide, like nifedipine also did not cause significant changes in duration of seizures but altered the hippocampal pO$_2$ profiles leading to increased baseline pO$_2$ and reduced severity of hypoxia following seizures (figure 4-4). The other anti-seizure drugs which were tested caused no significant changes in the durations of seizures as well as baseline and post-seizure hippocampal pO$_2$ profiles when compared to pre-treatment and their vehicle controls. Thus, hippocampal seizure durations, baseline oxygen levels and severity of seizure induced hypoxia were not significantly altered by these drugs. Like the most classic anti-seizure drugs, the putative anticonvulsant, bumetanide did not cause significant changes in the seizure durations and it neither prevented nor worsened the seizure induced hypoxia (figure 4-9). Effects of the ASDs on seizure durations, hippocampal oxygen levels and seizure induced hypoxia are summarized in figure 4-10 below.
Figure 4-10: Effects of ASDs on seizure induced hypoxia and seizure durations
Figure 4-10 shows the effects of nifedipine (green), classic anti-seizure drugs (orange) and the putative anti-seizure drug, bumetanide (yellow), on seizure durations, hippocampal baseline pO$_2$ levels and severity of seizure induced hypoxia. The presented results are the mean differences of drugs’ effects relative to vehicles’ effects. None of the drug significantly changed the seizure durations (panel A). Ethosuximide is the only ASD which, like nifedipine caused significant increase in the baseline hippocampal pO$_2$ (panel B) and significant reduction in the severity of seizure induced hypoxia (panel C).

* denotes $p < 0.05$ but $> 0.01$, ** denotes $p < 0.01$. 
4.4 Discussion

Brain ischemia/hypoxia causes brain damage (Gale and Hopkins, 2004; Malhotra et al., 2001; Mattiesen et al., 2009; Vintila et al., 2010) and frequently leads to either transient or permanent impairment of sensory, cognitive or motor functioning (Davies et al., 2013; Hernandez et al., 2003; Ibrahim et al., 2012; Oddo et al., 2003; Sanchez-Carpintero and Neville, 2003; Smith et al., 2002; Vintila et al., 2010; Yan et al., 2011). Therefore determination of effectiveness of ASDs in preventing development of seizure induced hypoxia is important in selecting ASDs to be given to patients with epilepsy. The current study is the first to show that ethosuximide is the only ASD which causes significant reduction in the severity of hypoxia that is expressed post-seizures, the effect similar to that of calcium channel blockers such as nifedipine. The rest of the ASDs (phenytoin, phenobarbital, valproate, levetiracetam, topiramate and lamotrigine) and the high loop diuretic bumetanide essentially did not alter the severity of seizure induced hypoxia and any changes noted with them could be due to their vehicles’ effects, as the post-drugs and post-vehicles changes were almost similar. Furthermore, ethosuximide, like nifedipine which had a positive effect on preventing SISHE caused a significant elevation of the hippocampal pO\textsubscript{2} before seizures were induced.

Reduction of SISHE severity caused by ethosuximide is likely due to drug’s inhibitory effect on cerebral vascular smooth muscles leading to vasodilation and increased cerebral blood flow. Ethosuximide has been shown to activate Ca\textsuperscript{2+} - dependent - K\textsuperscript{+} channels (Kristev et al., 1994; Velkova et al., 1995). Activated Ca\textsuperscript{2+} - dependent - K\textsuperscript{+} channels cause K\textsuperscript{+} efflux which leads to hyperpolarization and relaxation of smooth muscles and thus vasodilation (Kristev et al., 1994; Velkova et al., 1995). Reduction in the severity of SISHE caused by ethosuximide is unlikely be related with the drug’s inhibitory effects on neuronal excitability, as the present study
has also shown lack of significant changes in seizure durations following ethosuximide treatment.

LTG did not reduce or prevent development of SISHE, the finding which is contrary to expectations as previous studies indicated that LTG improves recovering from ischemic strokes. Focal and global cerebral ischemia models have shown that LTG promotes recovery from cerebral ischemic damage (Crumrine et al., 1997; Graham et al., 1993; Smith and Meldrum, 1995; Wiard et al., 1995). It is likely that improved recovering from stroke caused by LTG is due to neuroprotective effect of this drug, rather than changes in cerebral blood flow and oxygen delivery. Recovery from ischemic stroke caused by LTG is probably due to the drug’s effect in limiting the presynaptic release of excitatory amino acids neurotransmitters (Leach et al., 1986). In one experiment LTG was found to prevent hypoxia induced hippocampal neuronal damage; the neuroprotective effect which was associated with decreased the asphyxia-induced hippocampal tissue levels of glutamate and aspartate (Papazisis et al., 2008), the neurotransmitters which are excitotoxic when act via NMDA or non-NMDA receptors (Choi et al., 1988; Rothman and Olney, 1986). These neurotransmitters are thought to be involved in the development of cerebral infarction (Park et al., 1988). Failure of LTG to reduce the severity of SISHE could also be due to drug’s effect on respiratory functions. Although changes in breathing were not noted during experiments, LTG is known to impair peripheral chemoreceptor neuronal response to acute hypoxia where it reduces the rate of firing of the chemoreceptor afferent neurons (Faustino and Donnelly, 2006).

The findings that PHT and PB lack effects on severity of SISHE are in contrary to expectations since it has previously been reported that PHT and PB worsen ischemic strokes (Brailowsky et al., 1986; Goldstein, 1995; Goldstein et al., 1990; Hernandez and Russell, 1992).
It is likely that worsening of ischemic strokes caused by these ASDs is due to mechanisms unrelated to alterations of cerebral blood flow and brain oxygen supply or effects require long term use of these ASDs. Studies have shown that PHT cause muscle cells hyperpolarization and thus muscle relaxation (Beech et al., 1988; Minaker et al., 1989). Therefore it was anticipated that, this drug will cause increased cerebral blood flow due to cerebral vasodilation and thus minimize or prevent development of SISHE. However, the current study has found PHT lacked effect on SISHE. It is possible that PHT affects skeletal muscles with only little or no effect on vascular smooth muscles and thus failure to alter cerebral perfusion and cerebral oxygen level or its effects on muscle activity requires chronic administration. Only acute effects were tested in the current experiments.

None of the ASD caused significant change in seizure duration. Valproate, LEV and topiramate caused a non-significant reduction in seizure durations while post-PHT, post-PB and post-LTG seizure durations were slightly longer than pre-treatment values and post-ethosuximide and post-bumetanide seizure durations were almost similar to pre-treatment values. These findings are in contrary to the previous studies which have demonstrated shorter seizure durations following ASD treatments (Borowicz et al., 2003; Gilbert et al., 2001; Gilbert et al., 2002; Lösch et al., 1998) reflecting ASDs’ effects on seizure propagation. Gilbert et al. (2001) found significant reduction in ADDs by PHT in amygdala kindled and non-kindled guinea pigs. Other studies in amygdala kindled animals have also reported shortened ADDs in response to PB, valproate (Gilbert et al., 2002), LEV (Lösch et al., 1998) and topiramate (Borowicz et al., 2003) treatment. The differences between the current study and previous studies are likely due to differences in the stimulating currents and sites of seizure origin. The previous studies used smaller current intensities around the seizure threshold while in the current study the
stimulating current was far supra-threshold (500 µA). Additionally, all the previous studies which showed shortened seizure durations, the kindling were done in the amygdala while in the current study stimulation were done in the dorsal hippocampus, a place where there has been minimal effects of ASDs. For example PHT and valproate were found to lack significant effects on hippocampal generating seizure durations and carbamazepine only at high dose produces slight effect on hippocampal seizure durations (Emori and Minabe, 1990).

Generally it can be concluded that, ASDs by inhibiting neuronal activity do not alter the severity of seizure induced hypoxia. Only nifedipine and ethosuximide with significant effects on vascular smooth muscle activity directly can alter the development and severity of SISHE. Our findings that most ASDs do not prevent expression of SISHE or reduce its severity, is important information for clinicians prescribing these drugs for their patients with epilepsy.
CHAPTER FIVE

SUMMARY OF THE FINDINGS AND GENERAL CONCLUSION

5.1 Introduction

The main objective of this thesis was to examine the reorganization of motor maps following the use of anti-seizure drugs (ASDs) and the likelihood of the same drugs to prevent or reduce the severity of seizure induced hypoxia. The thesis also investigated whether the putative anti-seizure, bumetanide, has effects in adult brain neurons or not. Bumetanide was tested because it has shown promising role in controlling neonatal seizures and in one study it was found to reduce seizure frequency in adult patients.

Experiments conducted to answer these objectives are described in chapters 2-4. All experiments were conducted using male rats. Prior to experimental studies which are presented in chapters 2-4, I gave a detail description of how seizures and epilepsy alter motor maps expression. I also highlighted that, despite of long history of motor maps study using human and animals, the study of changes in motor maps expression following the use of ASDs has not been given much attention. Furthermore, I described a recently discovered phenomenon, long lasting severe brain hypoxia which develops post-seizures. This phenomenon is speculated to be, in part, responsible for neurological symptoms and signs among patients with epilepsy. In this last chapter of the thesis, I give the summary of the main findings from experiments I conducted. I then describe in brief the limitations of this study and outline what I feel are important future research projects to be done. I also give a brief description of my personal experience on the laboratory work and PhD training in general.
5.2 Summary of the Study Findings and General Discussion

Chapter two investigated acute effects of five classic ASDs on young adult rats motor maps expression. Using the high-resolution intracortical microstimulation (ICMS) technique, treatment with ethosuximide, valproate, levetiracetam and topiramate resulted into smaller forelimb maps while phenytoin caused no significant alteration in the forelimb map size. Further results showed that, all the ASDs mentioned above increased the current required to evoke both the forelimb and non-forelimb movements. I related the reduction in forelimb maps size and increased movement thresholds (MTs) observed post-anti-seizures to the inhibitory effects of these drugs on cortical-motor neurons and sensorimotor neocortical network output activity, related to movement generation (Borowicz et al., 2003; Gilbert et al., 2001; Gilbert et al., 2002; Löscher et al., 1998; Lothman et al., 1991). Forelimb map hypotrophy caused by ASDs also suggests that ASDs inhibit synaptic transmission at horizontal interconnected fibers, since map sizes in the neocortex are related to horizontal fibers synaptic transmission. Previous experiments showed that, larger motor maps are associated with synaptic potentiation (Henry et al., 2008; Teskey et al., 2008), suggesting that long term synaptic depression leads to smaller maps. I linked the increased MTs to the inhibitory effects of these drugs on cortical-motor neurons, whose axons forms corticospinal or corticobulbar tracts (Sohn et al., 2001). Increased MTs could also be a result of these drugs inhibiting the spinal alpha-motor neurons innervating the trunk or limb muscles, or cranial nerves which innervate muscles of facial expression. The ASDs could also have raised MTs by inhibiting the skeletal muscle directly or interfering with neuromuscular junction transmission.
Chapter three aimed to determine whether bumetanide has anti-seizure activity in adults or not. The study tested the effect of bumetanide on seizure parameters namely afterdischarge thresholds (ADTs), afterdischarge durations (ADDs) and seizure stage as well as on motor maps expression in young adult rats. Bumetanide significantly increased ADTs and reduced seizure stage without significant changes on ADDs. Bumetanide also increased both the forelimb and non-forelimb MTs in adult naïve and kindled rats without significantly altering the size of the forelimb area representation in the motor cortex. Electrophysiological studies were also done to test the effect of bumetanide on layer V pyramidal neurons of postnatal rats. Bumetanide significantly inhibited the activity of these neurons. These findings, collectively suggest that bumetanide has significant anti-convulsant activity in adult neurons. Bumetanide is a well-known NKCC1 blocker, the effect which eliminates GABA depolarizing effect on immature neurons. Therefore, our research findings suggest that the anti-convulsant properties of bumetanide are not merely due to its inhibitory effect on NKCC1 since the NKCC1 expression diminishes with aging (Dzhala et al., 2005; Payne et al., 2003; Wang et al., 2002). Therefore, there must be other unknown mechanisms which make bumetanide control seizures.

Chapter four investigated the roles of several ASDs on prevention of severe seizure induced hypoxic episode (SISHE). Ethosuximide elevated brain baseline oxygen levels and effectively prevented development of SISHE. The rest of ASDs which were tested (phenytoin, phenobarbital, valproate, levetiracetam, topiramate and lamotrigine) as well as the putative ASD; bumetanide did not cause significant alteration of brain baseline oxygen level and severity of seizure induced hypoxia. Different from other ASDs, ethosuximide has significant effect on smooth muscles. It causes hyperpolarization of smooth muscles (Kristev et al., 1994; Velkova et al., 1995). Hyperpolarization of cerebral vascular smooth muscles cause vasodilation (Velkova et
al., 1995) and increase cerebral blood flow and oxygen supply. Therefore prevention of SISHE by ethosuximide is thought to be due to its inhibitory effects on vascular smooth muscles rather than inhibition of neuronal activity.

5: 3 General Conclusion

Based on the study findings, I can conclude that ASDs using various mechanisms to inhibit either the motor neurons and/or skeletal muscles, or inhibit synaptic or neuromuscular junction transmission raise the thresholds for evoking movements. Neurons inhibited by ASDs may include cortical-motor neurons or neurons which innervates the skeletal muscles. Rising of MTs by ASDs can directly be linked to their anti-seizure activity. Map hypotrophy which was observed in the current study is related to reducing excitability of cortical motor neurons particularly in the horizontal interconnected fibers by the ASDs. This study has also revealed that bumetanide has anti-seizure effect in adult rats and therefore suggests that the anti-seizure effect of this drug does not exclusively depend on inhibiting NKCC1, but also depends on other unknown mechanisms. Furthermore, I can conclude that ASDs by inhibiting neuronal functioning neither prevent development of seizure induced hypoxia nor make it worse, which is important information for clinicians.

5: 4 Study Limitations

Due to limited time, I assessed only the acute effects of ASDs on motor maps expression and on prevention of SISHE. Since the use of ASDs by patients with epilepsy is on long term basis, it would be appropriate to test the long term effects of ASDs on motor maps expression and their potential role in preventing SISHE. Although there might be different effects following
chronic administration on ASDs before testing their effects on motor maps expression, the results following acute administration provides important information.

People with normal brain neuronal functioning do not use ASDs; only people whose brains have epileptic foci use ASDs. Therefore, it was important for me to test effects of ASDs on motor maps expression using rats that had seizures and motor map reorganization as was done for bumetanide. Nevertheless, the findings that ASDs raised MTs and caused forelimb map hypotrophy in naïve rats give important information which may be used to predict how the use of ASDs by patients with epilepsy affect health cortical brain regions that control movements. Furthermore, the question of how ASDs affect motor maps expression in patients with epileptic foci is being dealt by another student in our laboratory who is investigating the effects of ASDs on motor maps expression in kindled rats. He has found similar results as mine from naïve rats.

5.5 Future Direction

I have shown that ASDs affect MTs and body parts representation in the motor cortex, I therefore suggest a study to investigate how ASDs affects skilled movements. In areas with limited modern laboratory facilities, this can be performed by comparing skills movement of patients with epilepsy who are on various ASDs and those who are not on any medication.

This thesis has provided evidence that bumetanide has anti-seizure activity in adult rats, and previous study in adult patients with temporal lobe epilepsy has shown that bumetanide reduces seizure frequency (Eftekhari et al., 2013). Since this drug has shown promising role in managing adult seizures, there is a need to carry more studies testing efficacy of bumetanide in managing seizures in adults. In this thesis I have also shown that bumetanide hyperpolarizes post-natal rats’ neurons, and reduce their rate of firing. There is a need to carry electrophysiological studies testing bumetanide using slices prepared from adult rats’ brains.
Since ASDs except ethosuximide have shown to be ineffective in preventing severe seizure induced hypoxia, while calcium channels blockers such as nifedipine effectively prevents SISHE development there is a need to test the use of agents which prevents SISHE in combination with ASDs in preventing neurological symptoms which occur in patients with epilepsy.

Lastly, I would like to mention that this work has been hard to me but very interesting. I felt it difficult initially, but with coaching from my supervisor and colleagues in the laboratory, the work became more interesting and with time it started getting easier. Generally, the whole exercise of developing a research proposal, conducting experiments, analyzing data and presenting results and a discussion has transformed me a lot. This has put me in a capacity to develop research proposals, analyze data and write research reports with minimal assistance from a senior researcher. Due to limited laboratory equipments at my home country, it is likely that I will not be able to carry out laboratory experimental studies similar to the ones I conducted for my thesis. Nevertheless, I will be in a position to design epidemiological and clinical studies related to epilepsy and the use of ASDs to start with. This training has also made me capable of easily noting mistakes or errors in someone’s research proposal or scholarly report. This remarkable experience will also enable me to give assistance to junior trainees developing research proposals, analyzing data as well as writing organized research reports. To my institution, this will enable me to supervise and examine thesis for students in our undergraduate and master programs.
REFERENCES


representations following 6-hydroxydopamine administration in rats. *Exp Neurol*, 231, 82-90.


176


180


